Developing Immunotherapy Strategies for Cancer Treatment

Thesis

UNRESTRICTED

DEVELOPING IMMUNOTHERAPY STRATEGIES FOR CANCER TREATMENT

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Dedicated to my parents

Nontas and Areti.

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Abstract

The goal of this thesis was to develop potent tumour cell vaccines that can be used as an effective strategy for cancer treatment. Allogeneic tumour cell vaccines hold great promise for cancer treatment. Cytokine modification has been proven effective in enhancing the immunogenicity of tumour cell vaccines. This work here shows that modification of the allogeneic K1735 cells to express the immunostimulatory cytokines GM-CSF, IL-12 or IFN-γ, however, was not effective in augmenting the immunogenicity of the vaccine and irradiated cytokine-modified vaccines could not protect animals from autologous tumour challenge. These data are in agreement with recent literature. Similar results were obtained when K1735 cells were modified to express heat shock proteins. Analysis of the injection site of irradiated K1735 vaccine cells demonstrated that clearance of the K1735 cells from the injection site correlated well with the induction of proinflammatory cytokines and infiltration by non-specific effector cells such as NK cells.

FMG-induced fusion was next investigated as a novel and effective way of releasing tumour antigens from allogeneic vaccine cells in an immunostimulatory fashion. Expression of the Vesicular Stomatitis Virus G glycoprotein by K1735 or B16 cells lead to the formation of extensive syncytia *in vitro*. Vaccination of mice with irradiated 2-day fusing allogeneic K1735 or syngeneic B16 cells showed no significant therapeutic benefit relative to irradiated unfused cells. Interestingly, vaccination of mice with a hybrid K1735/B16 fusing vaccine repeatedly lead to significant protective and therapeutic immunity against a B16 challenge. Overall, VSV-G mediated syncitial death was shown to be a highly immunogenic event that promotes the induction of potent specific antitumour immunity in the context of allogeneic vaccines. The mechanism of immunogenicity of fusing hybrid vaccines was investigated.

Generation of long-term human allogeneic fusing tumour cell vaccines for translation of this work into clinical application was next investigated. A human melanoma cell line stably transduced with the gene for a hyperfusogenic form of the Gibbon ape leukemia virus FMG gene was generated. The Tet-On system of transcriptional control was employed to control gene expression and thus GALV-mediated fusion. Very high levels of fusion could be obtained following the addition of doxocycline in culture. This cell line could form the basis of human melanoma fusing vaccine.

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Glossary of Abreviations

AFP	alpha fetoprotein
APC	antigen presenting cell
BSA	bovine serum albumin
CD	cytosine deaminase
cDNA	complementary deoxyribonucleic acid
CEA	carcinoembryonic antigen
CMV	cytomegalovirus
cpm	counts per minute
CTL	cytotoxic T lymphocyte
dNTPs	dATP, dCTP, dGTP, dTTP
dH_2O	distilled water
DC	dendritic cell
DEPC	diethylpyrocarbonate
DMEM	Dulbecco's modification of Eagle's medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
Dox	doxocyclin
DTH	delayed type hypersensitivity
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
FMGs	fusogenic membrane glycoproteins
FRAP	FKBP12-rapamycin associated protein
FRB	FKBP-rapamycin binding
g	relative centrifugal force
gr	gramme
GCV	ganciclovir
GALV	gibbon ape leukemia virus
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	granulocyte macrophage colony stimulating factor
HA	hemagglutinin
HPV	human pappiloma virus
HSPs	heat shock proteins
HSV	Herpes simplex virus
ICAM	intracellular adhesion molecule
IFN-?	interferon-_
IL-	interleukin-
KIR	killer immunoglobulin receptor
L	litre
LAK	lymphokine-activated killer (cell)
luc	luciferase
M	molar
MART-1	melanoma antigen associated by T-cells-1
MHC	major histocompatibility complex
µl	microliter
MLV	murine leukaemia virus

MMR	macrophage mannose receptor
MoMLV	moloney MLV
MOPS	-3-(N-morpholino) propanesulphonic acid
mRNA	messenger ribonucleic acid
NCR	natural cytotoxicity receptor
NK	natural killer (cell)
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PRR	proline rich region
PSA	prostate specific antigen
PSMA	prostate specific membrane anigen
RBP	receptor binding domain
RNA	ribonucleic acid
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute 1640 (cell culture medium)
rtPCR	Reverse transcriptase-PCR
rtTA	reverse tetracycline transactivator
SU	surface unit
TAA	tumour associated antigen
TAE	Tris-acetate-EDTA buffer
TAM	tumour associated macrophages
TAP	transporter associated with antigen presentation
TCR	T cell receptor
tet	tetracycline
tetR	tetracycline repressor
TGF	Tumour growth factor
Th	T helper
tk	thymidine kinase
TIL	tumour infiltrating lymphocyte
TM	transmembrane
TNF	tumour necrosis factor
TRE	tetracycline responsive element
Tris	tris[hydroxymethyl] aminomethane
TRP-_	tyrosine related protein-_
TSP	tissue specific promoter
tTA	tetracycline transactivator
tTS	tetracycline transcriptional silencer
X-gal	5-bromo-4-chloro-3-indoyl-b-D-galactosidase
VSV	vesicular stomatitis virus
v/v	volume per volume
w/v	weight per volume

Chapter 1

INTRODUCTION

1.1 Cancer Immunotherapy

The concept of employing the immune system to effectively eliminate any insult to the human body, be it a bacterial pathogen, a virus, or cancer, has been continuously explored over the course of the last two centuries. Since first described in the late 1790s by Jenner, the development of effective immunoprophylactic vaccines has flourished to become one of the most successful types of medical intervention. In contrast to the widespread success of immunoprophylactic vaccination for infectious diseases, imunotherapeutic intervention for the treatment of cancer has been less successful. One of the first documented reports of tumour immunotherapy in humans was in the late 18^{th} century, when W. Coley used bacterial extracts to boost the immune system against tumour cells in cancer patients. This approach was based on observations of tumour regression following incidental bacterial infections (Old 1992). Interest in the field of cancer immunotherapy has dramatically oscillated over the course of the last century between overenthusiasm for the applicability of vaccines in inducing potent anti-tumour immune responses and severe pessimism for the validity of such an approach. The immense complexity of the immune system and the diversity of mechanisms underlying tumour development are the principal causes behind the relatively slow progress in the development of effective, clinically relevant imunotherapeutic vaccination approaches for cancer therapy.

Present enthusiasm for cancer immunotherapy has come as a direct result of advances made primarily over the last two decades in understanding the biology of the immune system, identifying cellular and molecular components crucial to immune recognition, priming and regulation, and new insights in tumour biology. Critical technological advances in the field of vector development and gene therapy have also made it possible to consider anti-tumour vaccination as a pragmatic approach to cancer therapy.

Most cancer cells have multiple genetic defects. Some gene therapy approaches for cancer treatment aim to correct such genetic defects and thus reverse malignant cells to a non-malignant phenotype. However, since most cancer cells have an accumulation of multiple defects, and restoration of the phenotype must be achieved in every single cell in a tumour mass, successful corrective gene therapy is faced with great challenges. These challenges arise from limitations in identifying the whole spectrum of important genetic defects in each tumour and the current lack of efficient gene delivery strategies for targeting and transducing all malignant cells. At present, corrective gene therapy can not address the issue of effectively targeting metastatic lesions commonly present in cancer patients or the issue of genetic diversity between different lesions in the same patient.

Immunotherapy of cancer on the other hand is primarily focused in eradicating cells with a malignant phenotype, rather than attempting to revert them to a normal state. Many gene transfer strategies have been developed for the *in vivo* or *ex vivo* introduction of cytotoxic genes or immunostimulatory genes, such as cytokines, into tumour cells or cells of the immune system. Here the aim is to directly kill tumour cells and/or augment the immune response against them, resulting indirectly in tumour cell death.

1.2 Tumour Immunology

A landmark in the development of immune-based strategies for cancer treatment has been the realisation that tumour cells express a range of tumour associated antigens (TAA) that can present targets for immune cell activation and immune-mediated elimination of malignant cells. Tumour infiltrating lymphocytes (TIL) isolated from the stroma of growing tumours in mice could be activated to recognise and eliminate tumour cells in *in vitro* assays (Barth, Bock et al. 1990). Adoptive transfer of these TIL to tumour bearing animals could mediate regression of established disease (Spiess, Yang et al. 1987). Similar observations have been made in human studies where administration of autologous TIL plus interleukin-2 (IL-2) to patients with metastatic melanoma resulted in regression of established invasive disease in some individuals (Muul, Spiess et al. 1987; Rosenberg, Packard et al. 1988). Identification

of such tumour-reactive TIL has provided a rational basis for the development of strategies aimed at inducing or further augmenting immune responses to tumours.

1.2.1 Identification Of TAA

Identification and *ex vivo* expansion of tumour-reactive lymphocytes from patients provided a useful reagent for the molecular identification of cancer antigens. Using lymphocytes from a melanoma patient, Boon and colleagues identified the MAGE family of genes, which were expressed on a subset of cancers as well as on gonadal tissue (van der Bruggen, Traversari et al. 1991). A tumour reactive cytotoxic T lymphocyte (CTL) clone exhibiting cytotoxic activity against autologous tumour cells in *in vitro* assays was expanded from the peripheral mononuclear cells of this patient and used to identify an antigen-loss variant of the original tumour that was not lysed by the CTL clone. The genes encoding the antigens recognised by the CTL clone were then identified by modifying the antigen-loss tumour variant with DNA libraries from the original tumour and testing for restoration of recognition by the CTL clone. The gene encoding the target antigen recognised by the CTL could then be isolated. An improved strategy for identifying target genes encoding relevant TAA involves the generation of tumour-derived cDNA libraries, the transient transfection into 293 or COS cell lines expressing the appropriate Major Histocompatability Complex (MHC) gene, and determination of recognition and lysis by autologous CTL or TIL. When positive pools are identified they are subcloned and individual genes responsible for conferring sensitivity to lysis by the CTLs or TIL are isolated. This technique was first successful in identifying the melanoma antigen tyrosinase (Brichard, Van Pel et al. 1993; Robbins, el-Gamil et al. 1994).

TAA can also be identified using a biochemical technique in which MHC Class I complexes from tumour cell lysates are immunoprecipitated and the bound tumour-specific peptides acid-eluted. The eluted peptides are then fractionated and peptide fractions are pulsed onto surrogate target cells, expressing the appropriate MHC Class I molecules. Specific TAA are identified by purifying and sequencing fractions conferring sensitivity to lysis by autologous CTLs or TIL (Van den Eynde, Peeters et al. 1995; Rosenberg 1997). A "reverse" method for identifying TAA has also been developed in which the sequence of proteins know to be overexpressed in tumour

cells is screened for the presence of MHC-binding motifs. Peptides within these sequences are synthesised and pulsed onto target cells expressing the appropriate MHC molecule and tested for their ability to mediate tumour recognition and lysis by CTLs and TIL. This approach was used to identify an HLA-A1-restricted epitope derived from MAGE-3 (Celis, Tsai et al. 1994).

Another approach of TAA identification has been developed recently and is based on the presence of antibodies against surface or intracellular cancer antigens in sera of cancer patients (Old and Chen 1998). In this approach recombinant cDNA expression libraries of human tumours are analysed serologically with autologous serum (Sahin, Tureci et al. 1997). Using this technique a range of novel as well as previously identified cancer antigens, such as tyrosinase and the MAGE antigens, have been described (Old and Chen 1998). A further technique for TAA identification has been the global expression profiling approaches such as SAGE (Martelange, De Smet et al. 2000; Riggins 2001). Comparing all genes expressed in tumours compared to their normal tissue of origin has identified patterns of gene expression common to tumour and distinct from normal cells. Further analysis of these data will identify multiple antigen targets that span multiple tumour types and MHC restrictions.

1.2.2 Tumour Antigens Recognised by T cells

Studies of antigens expressed in tumour cells, particularly melanoma, and recognised by autologous human T lymphocytes have established several important principles for human cancer immunotherapy (Rosenberg 2001). Firstly, cancer antigens can arise from various different proteins (normal differentiation antigens, cancer-testis antigens, viral antigens) and intracellular genetic events (alternative open reading frames, single base mutations, chromosomal rearrangement, post-transcriptional control of expression, intronic sequences, aberrant processing). Secondly, a given cancer antigen can contain epitopes that can be presented on many different MHC molecules. Tyrosinase for example contains epitopes that can be presented by HLA-A1, -A2, -A24, -B44, -DR4 and –DR15, while epitopes from gp100 can be presented by HLA-A2, -A3, -A24, -Cw8, -DR4, and -DR15. Thirdly, a given cancer patient can develop immune responses to multiple antigenic epitopes as demonstrated by observations of reactivity of the same polyclonal TIL population against multiple

different antigens. Finally, antigens from the same gene can be expressed in multiple different tumours. Antigenic epitopes deriving from the MAGE gene for instance have been identified in breast, lung, prostate, bladder and oesophageal cancers. Although the pool of known TAA is highly heterogeneous in composition, TAA recognised by autologous T cells can be generally classified into the following 5 categories (Table 1.1):

Table 1.1: Tumour Antigens Recognised By The Immune System

Category of antigen	Tumour
Unique tumour antigens	
β-catenin	Melanoma
caspase 8	Head and Neck
CDK4	Melanoma
Developmental antigens	
MAGE-1, -3, -4	solid tumours
GAGE-1, -2	solid tumours
BAGE	solid tumours
DAM	solid tumours
NY-ESO-1	solid tumours
Tissue-specific differentiation antigens	
gp100/pmel17	Melanoma
tyrosinase	Melanoma
MelanA/MART-1	Melanoma
TRP-1, -2	Melanoma
PSA, PSMA	Prostate
Oncogene & tumour suppresor gene products	
p53	50% solid tumours
Ras	Melanoma, pancreatic, colorectal
Her-2/neu	Breast, ovarian
Virally encoded antigens	
EBV, HPV, HTLV-1	Cervical, hepatoma, leukemia, lymphoma

1.2.2.1 Unique Tumour Antigens

Unique tumour antigens have been identified in tumours of individual patients and are usually the products of point mutations, gene rearrangements or changes in open reading frames that give rise to altered MHC Class I or Class II restricted T lymphocyte epitopes. An example of a unique tumour antigen identified in melanoma is an epitope generated from a point mutation of the gene for β-catenin (Robbins, El-Gamil et al. 1996). A single C to T base mutation gave rise to an HLA-A24 restricted nine amino acid peptide with over a million fold greater avidity for recognition by the patient's autologous TIL clone compared to the nonmutated 9-mer. A point mutation

in the cyclin-dependent kinase 4 (CDK4) gene gave rise to a peptide with altered binding affinity for HLA-A2, which constituted a novel T cell melanoma epitope (Wolfel, Hauer et al. 1995). Mutation of the CDK4 protein prevents it from interacting with the tumour suppresser gene and CDK4 inhibitor, $p16^{INK4a}$, therefore disturbing the normal cell cycle regulation machinery. An example of a TAA generated from alteration in an open reading frame is that of caspase 8 in a case of head and neck cancer (Old and Chen 1998). A mutation in a stop codon resulted in the extension of the normal open reading frame by 88 amino acids giving rise to novel T cell epitopes. Finally, another interesting type of unique tumour antigens are those arising from the idiotypic determinants of immunoglobulins expressed on clonal B cell malignancies, which have been explored as vaccines in studies of patients with B cell lymphomas (Kwak, Taub et al. 1995) (Hsu, Benike et al. 1996).

Most of the mutations or gene alterations giving rise to unique tumour antigens are thought to be critical contributors in the malignant phenotype of cells. The tumour specificity coupled with the patient specificity of unique tumour antigens renders them unlikely candidates for the development of generic vaccines with large-scale clinical application.

1.2.2.2 Developmental Antigens

Also known as embryonic antigens (expressed during embryonic development) or tumour shared antigens, developmental antigens are predominantly, but not uniquely, expressed by malignant cells. An interesting characteristic of these antigens is that their expression is shared by multiple types of cancers, making them attractive candidates for vaccination purposes. Expression of such antigens can also be shared with normal tissues, such as the testis or placenta, although the levels of antigen expression in normal tissues are usually very low. This apparent quantitative difference in expression levels between the different tissues sharing expression of developmental antigens can be exploited in the development of cancer vaccines. The MAGE-1 encoded antigen MZ2-E, which is an HLA-A1 restricted epitope (van der Bruggen, Traversari et al. 1991), is considered a prototype of molecularly identified human TAA recognised by autologous CTLs. MAGE-1 encodes an additional epitope restricted by HLA–Cw (antigen MZ2-Bb) (van der Bruggen, Szikora et al.

1994). MAGE-1, located on the X chromosome, is expressed in appoximately 40% of melanomas, as well as other tumour types including gliomas, breast cancer, head and neck, and non small cell lung cancer. Further cloning revealed a multigene family encoding several developmental antigens consisting of at least 12 MAGE genes and the closely related gene families of BAGE, GAGE, DAM (Boel, Wildmann et al. 1995) (Van den Eynde and Brichard 1995). Antigens expressed by these genes are found in melanoma and other cancers, as well as normal tissues such as placenta, testis and eye.

1.2.2.3 Tissue-Specific Differentiation Antigens

Tissue-specific differentiation antigens are nonmutated molecules expressed only by cells (normal or malignant) of the same lineage. Expression in transformed cells is generally upregulated compared to expression in normal counterparts. The best examples of this class of tumour antigens have been described in melanoma. Tyrosinase, gp100, melanoma antigen recognised by T-cells-1 (MART-1), tyrosinase related protein-1 and -2 (TRP-1 and TRP-2) are all proteins expressed in melanoma as well as normal melanocytes, and contain several MHC Class I or Class II restricted epitopes recognised by $CD8^+$ or $CD4^+$ T cells (Brichard, Van Pel et al. 1993) (Topalian, Rivoltini et al. 1994) (Cox, Skipper et al. 1994) (Robbins, el-Gamil et al. 1994). CTLs specific for such antigens that are capable of killing melanoma cells will also be able to kill normal melanocytes. Prostate specific antigen (PSA), prostate specific membrane antigen (PSMA), and PSH-P1 are similar examples of differentiation antigens expressed by normal prostate epithelia and overexpressed in prostate cancer and which can be recognised by specific CTLs. Differentiation antigens are good candidates for immunotherapeutic treatment of specific cancers arising in epithelial tissues not necessary for survival. In clinical studies in melanoma patients, adoptive transfer of TIL specific for melanoma differentiation antigens plus IL-2 resulted in regression of melanoma lesions (Rosenberg and White 1996). Clinical responses were often associated with the appearance of vitiligo, a depigmentation of the skin due to destruction of normal melanocytes. The appearance of vitiligo is associated with good prognosis and demonstrates that tolerance of the immune system to normal self antigens can be overcome. Of course, while less of

melanocytes can be tolerated, this may not be an acceptable side effect of immunotherapy for kidney or brain tumours, for example.

1.2.2.4 Oncogene and Tumour Suppressor Gene Products

Overexpression or point mutation of oncogenes or tumour suppressor genes can also give rise to TAA that can be recognised by $CD4^+$ or $CD8^+$ T cells. Oncogenes and tumour suppressor genes have central role in tumourigenesis and antigens arising from such molecules are very interesting targets for antitumour immunity. In addition to being recognised by specific TIL and CTL, these antigens can also be recognised by antibodies from the serum of patients.

The most commonly altered tumour suppressor gene in cancer is p53. p53 mutations are found in more than 50% of solid tumours. More than 120 different mutations have been identified in the p53 gene, most of which result in the generation and accumulation at very high levels of a non functional protein (Theobald, Biggs et al. 1995). The presence of any single p53 mutation in only a very small fraction of human cancers makes cancer vaccines aimed specifically at p53 mutations rather impractical (Pardoll 1998). Nonetheless, CTL can be generated against either human (Yanuck, Carbone et al. 1993) or murine (Noguchi, Chen et al. 1994) mutated p53 protein. Alternatively, CTLs specific for a wild-type p53 peptide can be created and adoptive transfer of these CTLs in animals bearing tumours overexpressing mutated p53 has been shown to generate a significant therapeutic effect (Vierboom, Nijman et al. 1997).

An oncogene that has been extensively studied as a target for anti tumour immunity is the Ras proto-oncogene (Disis and Cheever 1996). Three main activating mutations in the Ras gene (in positions 12, 13 and 61) have been identified in melanoma, pancreatic and colorectal cancers (Gaudernack 1996) giving rise to novel MHC Class I and Class II restricted epitopes recognised by $CD4^+$ and $CD8^+$ T cells (Fossum, Olsen et al. 1995). Antibody mediated responses to mutated Ras have also been identified in patients with gastrointestinal cancer. Her-2/neu, a membrane tyrosine kinase receptor, is an oncogene normally expressed in low levels in ovarian, mammary and other epithelia, and overexpressed in a number of carcinomas such as

breast and ovarian. Although not mutated, her-2/neu has been shown to elicit both antibody and T cell mediated responses in cancer patients (Peoples, Goedegebuure et al. 1995) and her-2/neu expressing tumour cell clones have been shown to be susceptible to lysis by autologous CTLs (Yoshino, Peoples et al. 1994).

1.2.2.5 Virally Encoded Antigens

As several different malignancies have been associated with specific viruses, interest in virally-encoded antigens as targets in the design of anti tumour vaccines has increased significantly. Although viral proteins can be directly oncogenic or indirectly contribute to the accumulation of a malignant phenotype in cancer cells, it is not know whether they are necessary for maintenance of the transformed state, an important consideration in the development of cancer vaccines. Human pappiloma virus (HPV) types –16, -18, -31 and –45 have been strongly associated with cervical carcinoma. Approximately 80-90% of cervical cancers express the E6 and E7 antigens of HPV, which bind to and inactivate the tumour suppressor proteins p53 and retinoblastoma respectively. Efforts have been made to identify specific epitopes from E6 and E7 that can be presented by MHC molecules and generate a specific CTL response (de Gruijl, Bontkes et al. 1996; Ressing, van Driel et al. 1996). Other viruses implicated in malignant transformation and which may express potential tumour antigens are hepatitis virus (hepatoma), Epstein-Barr virus (lymphomas and nasopharyngeal cancer), and human T cell leukemia virus-1 (leukemias). In addition to being potentially strong candidates for therapeutic cancer vaccines, virus-associated tumour antigens also present a great opportunity for the development of prophylactic vaccination strategies (Chang, Chen et al. 1997).

1.3 Immune Responses to Tumour Cells

The plethora of studies devoted to identifying antigens expressed in tumour cells that can function as tumour regression antigens clearly demonstrate that mediators of cellular immunity can not only recognise tumour antigens presented to them by MHC molecules but can also be licensed to kill cells displaying these target antigens. $CD8^+$ or $CD4^+$ T cells isolated from tumour-bearing patients expressing specific T cell receptors (TCR) can be activated *in vitro* upon ligation of the TCR to an MHC Class

I-, or Class II- peptide complex, respectively, and kill autologous tumour cells both *in vitro* and when adoptively transferred *in vivo* (Rosenberg, Spiess et al. 1986; Muul, Spiess et al. 1987; Spiess, Yang et al. 1987; Barth, Bock et al. 1990). In addition, antibody mediated responses against tumour cells have been described (Sahin, Tureci et al. 1997; Old and Chen 1998; Bremers and Parmiani 2000). However, in the majority of patients tumours thrive in the presence of an intact immune system, suggesting that the development of an effective antitumour immune reaction in patients is the exception and not the rule. Even in these cases where there is evidence of immuneresponsiveness, whether specific TIL or antibodies, the tumours progressively grow. Importantly, in most of the previously mentioned studies extensive *in vitro* conditioning with additional immune stimulatory factors, such as cytokines was required to activate T cells to produce effector functions (Herin, Lemoine et al. 1987) (Rosenberg, Packard et al. 1988). Understanding the mechanisms underlying immune responsiveness to tumour cells is therefore essential for designing strategies to modulate immune responses and effectively directing them against tumour cells.

The immune system is a highly complex and gracefully orchestrated network of cells and soluble factors. It is the fine-tuning of this system that determines the outcome of a reaction – stimulation, suppression, tolerance or ignorance. Two major branches constitute the core of host resistance against invading agents and tumours: *innate* immunity and *adaptive* immunity. Interaction between the two branches is very close and regulated by a variety of cells of the immune system and soluble factors (Belardelli and Ferrantini 2002).

1.3.1 Innate Immune Response

The innate immune system is traditionally considered as the first line of defence against invading pathogens such as bacteria and viruses. Increasing evidence however has revealed that cells of the innate immune response have critical roles in the host responses against malignant cells. Two of the main characteristics of innate immunity are a lack of immunological memory and antigen recognition via specific structurally and genetically conserved pattern recognition receptors. Key players of the innate immune system include macrophages, natural killer (NK) cells, neutrophils,

eosinophils and dendritic cells, although as will be described later, some of these cells play significant roles in the adaptive branch of immunity as well. Most of these cells recognise non-processed antigen, such as carbohydrates and nucleic acids of pathogens, via a spectrum of different pattern recognition receptors. Pattern recognition receptors can be expressed on the cell surface, in intracellular compartments, or secreted in the bloodstream and tissue fluids and examples include the macrophage manose receptor (MMR) expressed on the surface of macrophages (Fraser, Koziel et al. 1998), macrophage scavenger receptor (MSR) also expressed on macrophages (Pearson 1996) and Toll-like receptors expressed on NK cells, DCs and macrophages.

1.3.1.1 Natural Killer Cells

Key mediators of innate immunity known to have critical roles in anti-tumour immunity are NK cells. NK cells are large granular lymphocytes initially identified by their ability to kill tumour cells of different origins without prior activation. They arise in the bone marrow and comprise approximately 10-20% of the mononuclear cell fraction in peripheral blood. Unlike T cells, NK cells do not need to undergo thymal education for maturation (Dorshkind, Pollack et al. 1985) and do not express any TCR/CD3 complex on their surface (Lanier, Cwirla et al. 1986). They lack apparent antigen specificity and lyse tumour cells in a non-MHC-restricted fashion. Upon activation by tumour cells or cytokines such as IL-2 and IL-12, NK cells produce cytokines including IFN-γ and tumour necrosis factor -α (TNF-α) (Perussia 1991). Expression of cell surface receptors for IL-2, IL-12 and TNF-α on NK cells ensure that cytokines produced by activated NK cells can exert an autocrine effect, possibly enhancing their cytotoxic function. Lymphokine activated killer cells, predominantly consisting of activated NK cells, can be generated from the blood or from tumour-infiltrating lymphocytes upon IL-2 conditioning. These IL-2 activated NK cells have a broader specificity and enhanced ability to kill tumour cells.

The most important feature of NK cells for cancer immunity and the most important distinction between NK cells and T cells is their ability to recognise and kill tumour cells that lack MHC Class I on their surface. This makes them very attractive candidates for immunotherapy of tumours that have downregulated surface MHC

Class I expression to evade T cell recognition. For example, MHC Class I absence on lymphoma cells renders them susceptible to NK-cell mediated cytotoxicity (Karre, Ljunggren et al. 1986). Thus, Class I downregulation by transformation or infection may make cells susceptible to NK cell lysis (Raulet and Held 1995; Lanier, Corliss et al. 1997), while IFN-γ induced upregulation of Class I expression on tumour cells may protect them from NK mediated killing (Piontek, Taniguchi et al. 1985). Class I recognition by NK cells in humans is mediated primarily by killer immunoglobulin receptors (KIR). KIR are members of the immunoglobulin superfamily (Wagtmann, Biassoni et al. 1995) and can signal inhibition or activation upon MHC Class I binding depending on motifs present within their cytoplasmic tail. Interestingly, in mice MHC Class I binding NK receptors belong to a structurally different class of receptors, the type II lectins, capable of recognising sugar moieties on Class I molecules (Yokoyama and Seaman 1993).

The best characterised activation receptor on NK cells is the Fcγ receptor, CD16, which serves to bind soluble or cell bound IgG-complexes during antibody-dependent cell-mediated cytotoxicity and facilitate killing of the target cells (Takai, Li et al. 1994). Natural cytotoxicity receptors (NCR) are another type of activation receptors expressed on the surface of NK cells. Members of NCR include the recently identified NKp46, NKp30 and NKp44 (Moretta, Biassoni et al. 2000) all of which play important roles in the lysis of most tumour cell lines, as suggested by antibody-mediated receptor-blocking experiments. NKp46 high expressing NK cells have a greater ability to kill autologous, allogeneic or xenogeneic tumour cells than low NKp46 expressing NK cells. NKp44 is expressed uniquely on IL-2 activated NK cells, contributing to the increased ability of these NK cells to lyse a broad spectrum of tumour cells (Cantoni, Bottino et al. 1999).

In addition to their direct anti-tumour cytotoxic effects mediated by ligation of the inhibitory and activatory cell surface receptors on NK cells with MHC Class I molecules on target tumour cells, NK cells can have indirect, cytokine mediated antitumour effects. As mentioned earlier, NK cells can be activated to express a plethora of type 1 and type 2 cytokines, including IFN-γ, TNF-α, GM-CSF, M-CSF, IL-2, IL-3 and IL-8, plus chemokines. All of these may contribute to the activation of

$CD4^+$ T cells, $CD8^+$ T cells and DCs, thus influencing the development of the adaptive immune response to tumour cells (Trinchieri 1995; Kos 1998). NK cells can reciprocally respond to cytokines produced by cells of the adoptive immune response, suggesting the existence of a very close link between non-specific innate immune responses and antigen-specific T cell-mediated responses.

1.3.1.2 Macrophages

Macrophages are scavenger cells that are highly phagocytic and can express high levels of MHC Class II on their cell surface, thereby functioning as efficient APC able of stimulating $CD4^+$ helper T cells. Macrophages can determine the course of downstreem immune responses to tumours via cytokine secretion (Gough, Melcher et al. 2001). Often they are part of the cell infiltrates found in tumours. Expression of pro-inflammatory cytokines such as IL-12 by tumour associated macrophages (TAM) contributes to the activation of T cells as well as other effector cells such as NK cells and promotes recruitment of secondary immune cells, such as neutrophils, to the tumour site enhancing local cytotoxicity (Bartholeyns 1993). Like NK cells, macrophages play an important role in innate immunity by expressing several PRR and immunoglobulin Fc receptors which allow them to recognise pathogen-associated molecular patterns and mediate antibody-dependent cellular cytotoxicity respectively. The mechanisms of cellular cytotoxicity by activated TAM include secretion of reactive oxygen species (ROS), proteases, nitric oxide and cytotoxic cytokines such as tumour necrosis factor (TNF)-α and IL-1. ROS release by TAM however has been associated with irreversible inhibition of NK cell activity in some instances, leading to apoptotic NK cell death (Hellstrand, Asea et al. 1994; Kono, Salazar-Onfray et al. 1996).

Although macrophages can be highly effective at initiating antitumour immune responses, there is significant evidence that macrophages recruited to tumours are responsible for numerous pro-tumourigenic activities. Macrophages enhance vascular remodelling in the tumour environment and can also enhance the metastatic potential of primary tumours (Elgert, Alleva et al. 1998). In this way macrophages, while potentially a key component of an effective antitumour immune response, are most

likely to be found in patient tumours as active participants in the tumorigenic environment.

1.3.2 Adaptive Immune Response

The adaptive branch of host immunity is characterised by antigen specificity and immunological memory. Dendritic cells (DCs), $CD4^+$ T cells, $CD8^+$ T cells and B cells are the key mediators of adaptive immunity and the close interactions between these cells are crucial to the outcome of immune responses. T and B lymphocytes have the unique ability to create large numbers of clones expressing distinct antigen receptors, TCR, and immunoglobulin respectively, recognising antigens or peptides in a highly specific manner. Antigen specific cells do not have the ability to distinguish between structures that "require" an immune response and those that do not. Thus, selection of specific cells is critical to "elucidate" the repertoire of antigen specific clones to minimise immune responses in non-pathogenic circumstances. Moreover, antigen recognition by these antigen specific cells is not merely sufficient to instigate an effective antigen specific immune response. In fact, antigen recognition in the absence of subsequent signals is likely to lead to generation of antigen tolerance. It is cells of the innate immune system, particularly DC, which can efficiently provide the necessary signals to guide the development of an immune response to specific antigens.

1.3.2.1 Dendritic Cells

DC, the most professional type of APC, are an essential link between innate and adaptive immunity (Palucka and Banchereau 1999). Originally described by Steinman and colleagues (Steinman and Cohn 1973), DC represent a heterogeneous cell population encompassing multiple subsets with potentially distinct roles (Vremec and Shortman 1997). They reside in most peripheral tissues, particularly skin and mucosae, where they can continually take up antigens, process them into peptides and load them onto MHC Class I and II for presentation to T cells. There are at least three distinct subpopulations of DC, two within the myeloid lineage and one within the lymphoid lineage, and three stages of development, including precursor DC patrolling

through the blood and lymphatics, tissue resident immature DC and mature DC primarily found within secondary lymphoid organs (Palucka and Banchereau 1999).

Immature tissue resident DC are very efficient in capturing antigens from the local environment, but express very low levels of surface MHC and accessory molecules making them very inefficient T cell stimulators. Immature DC possess several mechanisms for antigen uptake particularly macropinocytosis (Sallusto, Cella et al. 1995), during which large volumes of extracellular fluid can be sampled for soluble antigens, receptor-mediated endocytosis (Guermonprez, Valladeau et al. 2002), which allows the uptake of macromolecules through coated pits, and phagocytosis of particles and microbes. Mouse and human immature DC express a plethora of surface receptors including Fc receptors (Esposito-Farese, Sautes et al. 1995; Fanger, Wardwell et al. 1996), which allow the uptake of Ig-coated particles, C-type lectin receptors such as DEC-205 (Mahnke, Guo et al. 2000) and MMR (Sallusto, Cella et al. 1995), scavenger receptors involved in uptake of apoptotic bodies (Platt and Gordon 1998), DC-specific ICAM3-grabbing non-integrin (DC-SIGN) (Geijtenbeek, Krooshoop et al. 2000) and Langherin (Valladeau, Ravel et al. 2000). Another class of very important receptors expressed by DC are the TLR responsible for recognition of pathogen-associated molecular patterns such as LPS, bacterial lipoproteins, CpG motifs present in bacterial DNA, and double stranded RNA (Medzhitov 2001).

To activate $CD8^+$ cytotoxic T cells, DC must present antigens in the context of MHC Class I molecules (**Figure 1.1.A**). Classically MHC Class I restricted peptides are products of endogenously synthesised proteins. Most proteins destined to be loaded on MHC Class I molecules are targeted for proteosomal degradation into short 8-10 amino acid long peptides by the ubiquitin (Ub)-proteasome pathway (Spataro, Norbury et al. 1998). The resulting antigenic peptides are transferred to the ER by the transporter associated with antigen presentation (TAP) and under the guidance of a loading complex composed of endoplasmic reticulum (ER) resident chaperones are loaded on newly synthesised MHC Class I molecules (Cresswell, Bangia et al. 1999). MHC Class I-peptide complexes are rapidly transferred to the plasma membrane where they become available for recognition by $CD8^+$ T cells. DC also have the ability to process and present exogenously derived antigens, such as tumour-derived antigens, through the MHC Class I pathway, in a process know as "cross-

presentation". Cross-presentation can occur either in a TAP-independent or a TAP-dependent manner (Yewdell, Norbury et al. 1999). The former mechanism requires that MHC Class I molecules are present in the endocytic pathway (Kleijmeer, Escola et al. 2001) and is generally insensitive to inhibitors of protein neosynthesis and inhibitors of the proteasome. The latter mechanism is better understood and has been demonstrated in several different experimental systems (Kovacsovics-Bankowski and Rock 1995; Rodriguez, Regnault et al. 1999). Cross presentation is believed to be critical to the development of protective antigen-specific immune responses against tumour cells that express very low to no MHC I molecules (Huang, Bruce et al. 1996). Additionally, cross presentation explains immune responses induced by allogeneic tumour cell vaccines that can not productively present tumour antigens on their MHC molecules to host T cells.

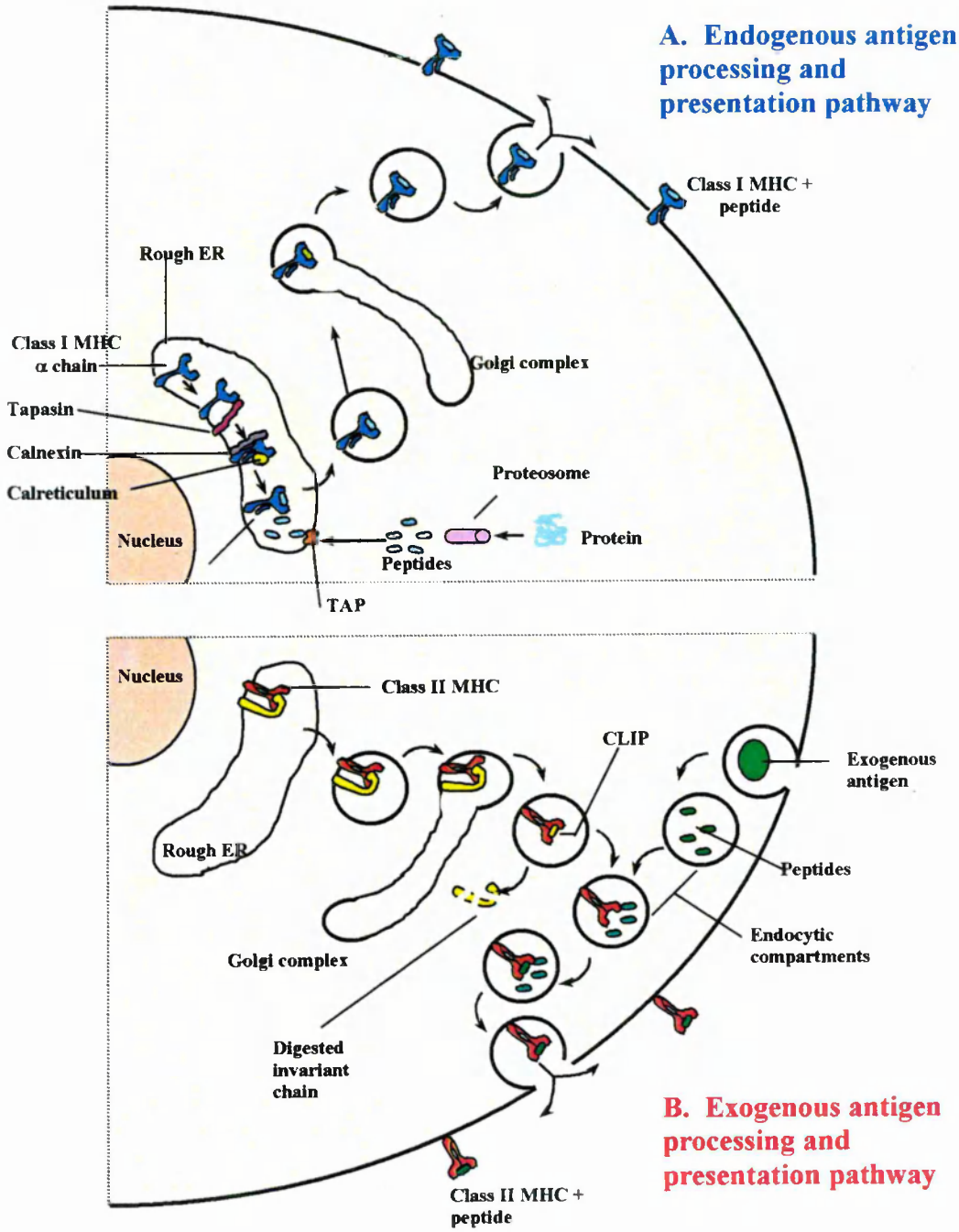


Figure 1.1: Antigen processing and presentation pathways.
A: endogenous antigens are degraded by the proteasome and following TAP-mediated transport to the rough ER they are loaded on free Class I MHC molecules with the help of the chaperoning molecules tapasin, calnexin and calreticulin. Binding of antigenic peptides to Class I MHC stabilises this complex and promotes release from ER and rapid transport to the plasma membrane. **B:** following uptake by the APC, exogenous antigens are degraded in endocytic compartments and are loaded on Class II MHC molecules in late endosomal compartments. Class II MHC molecules in the rough ER associate with the invariant chain (Ii) to prevent premature loading and to aid with transport to loading compartments. Ii becomes gradually degraded and its last remaining fragment, CLIP, becomes is replaced by an antigenic peptide. Class II MHC-peptide complexes are transported to the plasma membrane.

$CD4^+$ T cells recognise processed antigen presented on the surface of DC in the context of MHC Class II molecules (**Figure 1.1.B**). Exogenous antigens enter the endocytic pathway of the cells where they are degraded into 12-15 amino acid long peptides and are directed toward the MHC class II-rich compartments (MIICs), late endosomal structures abundant in immature DC (Watts 1997). MHC Class II molecules are synthesised in the ER where they associate with invariant chains (Ii) prohibiting their premature loading (Cresswell 1996). Transport signals present in the cytoplasmic region of the Ii chain guide MHC II-Ii complexes through the Golgi apparatus to endosomal and lysosomal compartments leading to the MIICs, where amidst an acidic, protease-rich environment, the Ii chain is degraded by several proteolytic enzymes and MHC Class II molecules become free for antigen loading. Antigen loading onto MHC Class II molecules is mediated by HLA-DM and HLA-DO products (Kropshofer, Hammerling et al. 1999). MHC Class II-peptide complexes are relocated to the cell surface, where they become available for binding to TCR on $CD4^+$ T cells (Cella Marina 1997; Santambrogio, Sato et al. 1999).

Antigen presentation through either of the two pathways of processing and presentation becomes extremely efficient once DC have received maturation signals. Mature DC express higher levels of MHC Class I and II molecules (Cella Marina 1997; Rescigno, Citterio et al. 1998), and have increased proteosomal activity in the lysosomal compartments resulting in more efficient degradation of antigens (Fiebiger, Meraner et al. 2001). Additionally, mature DC replace the standard proteasome with the immunoproteasome, which may enhance efficiency of processing of certain epitopes (Macagno, Gilliet et al. 1999; Morel, Levy et al. 2000). DC maturation can be triggered by a variety of factors. Maturation of DC is required for effective T cell activation as it leads to not only enhanced presentation of antigenic peptides but also to enhanced expression of essential costimulatory molecules on the surface of DC, as well as production of a variety of immune regulatory cytokines. Typically, microbial or viral products such as LPS, CpG DNA motifs and dsRNA can very efficiently induce DC maturation, and they do so via TLRs or other pattern recognition receptors on DC. Thus TLR provide a mechanism by which DC can link innate and adaptive immunity. Pro-inflammatory cytokines such as IL-1, GM-CSF and TNF-α also

trigger DC maturation, while IL-10 blocks maturation. DC have a unique ability of directing the outcome of an immune response, as demonstrated by the influence that the cytokine milieu generated by activated mature DC has. Release of cytokines such as IL-2, IL-12, IL-15, INF-γ, TNF-α and TNF-β can promote activation of T helper 1 (Th1) cells and development of cell mediated immune responses, whereas secretion of cytokines such as IL-4, IL-5, IL-6 and IL10 can promote activation of Th2 cells and humoral immune responses against tumours (Belardelli and Ferrantini 2002).

Migration and homing of DC to and from tissues or lymphoid organs is tightly regulated by chemotactic factors released by target tissues and by modulation of adhesion molecules expressed on the surface of DC (Palucka and Banchereau 1999). Differential expression of distinct chemokine receptors at various stages of DC maturation may play an important role in the migratory capacity of DCs (Sozzani, Allavena et al. 1998).

1.3.2.2 T Lymphocytes

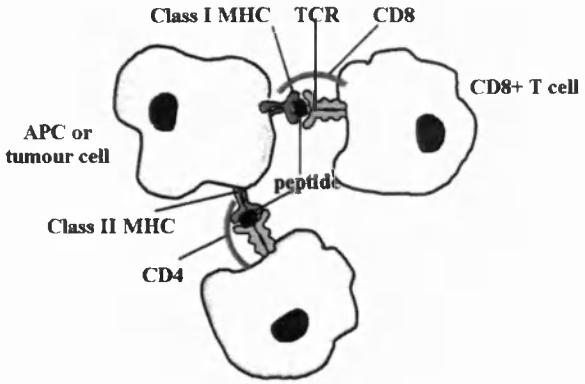
T lymphocytes are the crucial mediators of antigen specific cytotoxicity. As previously discussed naïve $CD8^+$ and $CD4^+$ T cells can recognise antigens presented by APC in the context of MHC Class I and Class II molecules respectively and following additional stimulation can be primed to respond against cells expressing this antigen on their surface. To avoid inappropriate and potentially harmful activation against antigens expressed by normal tissues, the immune system has developed several mechanisms for "de-sensitising" T cells against "self" antigens. In the thymus, developing T cells undergo a sequential process of thymic education (Mondino, Khoruts et al. 1996). Initially, in a process called "positive selection", T cells expressing a TCR with no affinity for self MHC are deleted, while those with moderate TCR reactivity to self MHC are stimulated to continue the maturation process. Surviving T cells subsequently undergo "negative selection" during which T cells carrying TCR with very high affinity for self peptides presented in the context of self MHC by cortical epithelial cells or APC are eliminated. This process, usually referred to as "central tolerance", ensures that circulating mature T cells can potentially react efficiently only against non-self antigens presented by self MHC molecules. It is clear however, that central tolerance is not absolute and T cells with

some reactivity to self-antigens presented by self MHC can escape in the periphery. This can be the case for antigens not expressed or not presented by APC in the thymus during T cell maturation, for "subdominant" epitopes of a self antigen that are possibly not adequately presented in the thymus (Cibotti, Kanellopoulos et al. 1992) or for antigens expressed only for a short period during development. Potentially self-reactive T cells existing in the periphery are believed to be kept in check by a mechanism referred to as "peripheral tolerance". Although the exact mechanisms behind peripheral tolerance are not well understood, it is believed that presentation of the self-antigens by self MHC molecules in the absence of any additional co-stimulatory or inflammatory signals (the case in normal physiological conditions) leads to T cell non-responsiveness (tolerance).

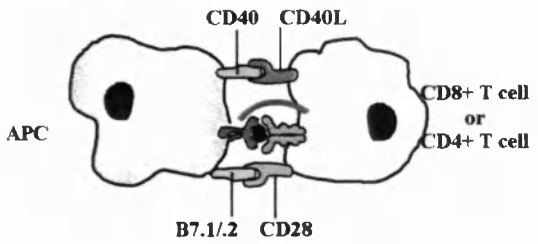
An alternative model to the self/non-self paradigm described above is provided by the "danger" model (Matzinger 1994; Fuchs and Matzinger 1996). This model proposes that it is not the nature of an antigen (self or non-self) that is the determining factor in the development of an antigen specific immune reaction, but rather that immune reactivity to any antigen can develop only if additional danger signals are provided in the same context as the antigen. If an antigen-associated signal is presented to a T cell in the absence of such danger signals immunological ignorance or tolerance will result (Gallucci and Matzinger 2001; Matzinger 2002). Indeed, studies of neonatal tolerance of foreign antigens in newborn mice are supportive of this model. In these studies, newborn mice can be made responsive to foreign antigens when antigens were given in appropriate doses, on appropriate APCs and with suitable adjuvants (Mahana, Guilbert et al. 1989; Forsthuber, Yip et al. 1996; Ridge, Fuchs et al. 1996). Although the danger model was originally proposed as an alternative to the self/non-self paradigm to explain immune responsiveness or tolerance to antigens presented to T cells, it is becoming increasingly evident that the two models need not necessarily be exclusive or incompatible with each other. The principles of the danger model have played a significant role in the design of antitumour vaccination strategies, where the goal is to break tolerance to self tumour antigens. Strategies in which cells such as DC are employed to provide cellular signals associated with danger, or genetic modification with genes that have the potential to directly or indirectly promote a dangerous or inflammatory environment in which the tumour antigens are presented, have been developed and applied with encouraging results.

Generation of effective tumour-specific immune responses involves a plethora of different cells and events that unfold in a highly synergistic and interactive fashion. Immune responses are generally regarded as having two major phases: the induction phase and the effector phase. Induction of a successful specific anti-tumour immune response requires that T cells receive a first signal (**signal one**) through ligation of TCR with MHC Class I-peptide complex in the case of $CD8^+$ T cells or MHC Class II-peptide complex in the case of $CD4^+$ T cells (**Figure 1.2.A**). Presentation of TAA can be direct by the tumour cell, in which case the TAA are presented on the cell surface in the context of MHC Class I molecules to $CD8^+$ T cells, or indirect by an APC such as DC, in which case both MHC Class I and Class II peptides can be presented to $CD8^+$ and $CD4^+$ T cells. This initial interaction between T cells and DC can be strengthened by high expression of several adhesion molecules such as integrins β1 and β2 and members of the immunoglobulin super familly. Direct presentation of TAA to $CD4^+$ T cells can occur when the tumour cells themselves express MHC Class II molecules, as is the case with tumours of lymphoid origin such as B cell lymphomas. Signal one on $CD8^+$ T cells is responsible for the antigen specificity of an immune response. A second, antigen non-specific, co-stimulatory signal (**signal two**) is required to sustain T cell activation. Signal two involves several molecules on APC which are recognised by T-cell surface receptors (**Figure 1.2.B**). The most well studied co-stimulatory molecules are the CD80 (B7.1) and CD86 (B7.2) on APC binding to CD28 on T cells and the analogous interaction between CD40 and CD40L. Finally, a polarisation/proliferation signal (**signal three**) is believed to be required for appropriate recruitment and proliferation of reactive lymphocytes (Kalinski, Hilkens et al. 1999). This signal is provided by cytokines or growth factors in order to recruit, polarise and propagate tumour reactive lymphocytes (**Figure 1.2.C**). Activated APC can provide such polarising signals. *In vitro* experiments with monocyte derived DC have shown that presence of IFN-γ during DC maturation induced DC to express high levels of IL-12 and drive $CD4^+$ T cells to the T helper1 (Th1) differentiation pathway. Th1 $CD4^+$ T cells secrete cytokines such as IL-2, IL-12, TGF-β and IFN-γ that promote cell mediated immune responses. In contrast, PGE_2 promotes low IL-12 production by DC and a Th2 driving capacity.

A. Signal One



B. Signal Two



C. Signal Three

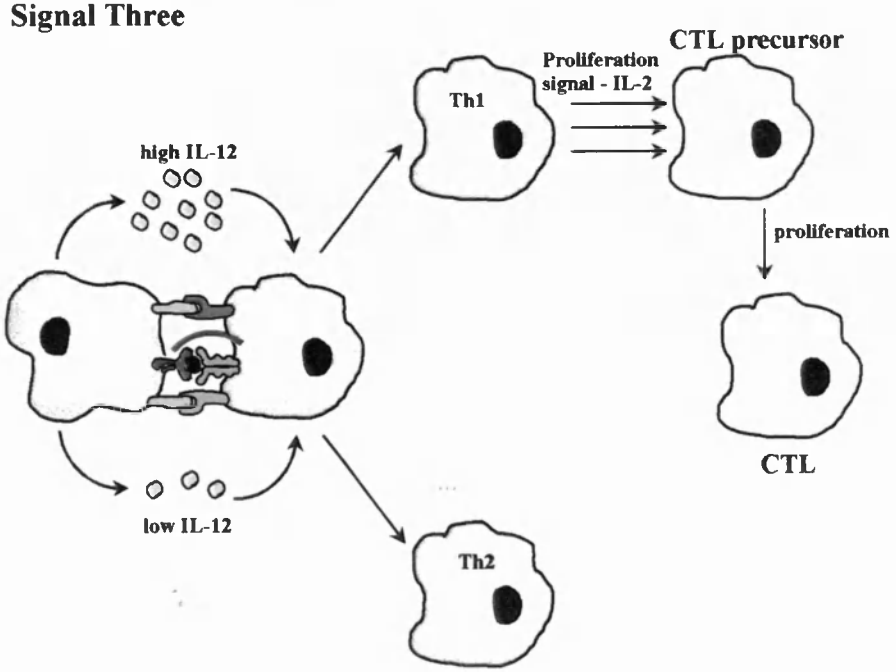


Figure 1.2: T cell activation.
A: *Signal one* of T cell activation involves ligation of the TCR on Tcells with an MHC-peptide complex on the APC. **B:** *Signal two* or *costimulation signal* involves ligation of costimulatory molecules such as B7.1, B7.2 or CD40 on the surface of the APC with the appropriate receptors such as CD28 and CD40L respectively on T cells. **C:** *Signal three* is a polirisation or proliferation signal provided by cytokines and growth factors. All three signals are believed to be essential for efficient T cell activation and proliferation.

Th2 $CD4^+$ T cells secrete cytokines such as IL-4, IL-5 and IL-10 that promote B cell-mediated humoral immune responses. It is now believed that distinct subsets of DC (lymphoid vs myeloid) can induce different class of immune responses (Th1 vs Th2). DC-stimulated $CD4^+$ T cells up-regulate CD40L that reciprocally activates and promotes maturation of DC via CD40 and this renders DC more potent stimulators of $CD8^+$ T cells. Activated T cells produce propagation/proliferation signals such as cytokines, promoting proliferation of specific CTLs and their effector functions. Specifically, IL-2, produced mainly by activated Th1 $CD4^+$ T cells, is the major helper proliferation signal for CTLs. The effector phase of an anti-tumour immune response is mediated by activated CTL, activated NK cells and specific antibodies. Importantly, in activated effector T cells signal one suffices for killing of the target cells, whereas signal one in the absence of co-stimulatory signals results in anergy of naïve T cells. This renders activated effector T cells able to kill target non-APCs that do not express appropriate costimulatory signals.

1.3.3 Cytokine Network Linking Innate and Adaptive Antitumour Immunity

Communication between cells of the immune system, including APC and lymphocytes, during the course of an immune response is mediated by cytokines. The type of host antitumour response is determined by the cytokines present in the tumour milieu. Immunostimulatory cytokines can induce or enhance antitumour immunity, whereas immunosuppressive cytokines can impair the host antitumour response. Cytokines linking the innate and adaptive branches of immunity include type I IFN, GM-CSF, IL-12, TNF-α, IL-15 and IFN-γ. Cells of the innate immune system such as macrophages and DC produce a number of these cytokines upon stimulation by pathogens or danger signals and thus directly and indirectly affect cells of the adaptive immune response.

GM-CSF is mainly produced by monocytes and macrophages and has a wide range of effects on cells of the innate and adaptive immune systems. It promotes expansion and activation of APC such as macrophages to produce other immunostimulatory cytokines such as IFN-γ, IL-12 and IL-15, which have an effect on cells of the innate immune system such as NK cells and induces migration of DC to lymph nodes (Mellstedt, Fagerberg et al. 1999). GM-CSF induces chemotaxis plus phagocytic and

cytotoxic activity of neutrophils and macrophages, and it has a strong effect in the induction of cell-mediated immunity by stimulating naïve T cells. The immunomodulatory effects of GM-CSF have made it an attractive candidate for use in antitumour vaccinations. GM-CSF can also enhance antibody-dependent cell-mediated cytotoxicity and amplify humoral immune responses (Belardelli and Ferrantini 2002). GM-CSF-mediated activation of monocytes however can also lead to production of an immunosuppresive cytokines, leading to suppression or down-modulation of immune responses (Mellstedt, Fagerberg et al. 1999).

IL-12, produced by activated APC, particularly DC, can promote the development, growth and cytotoxic activity of NK cells and induces production of IFN-γ by NK cells, DC, macrophages and T cells. IL-12 is believed to skew the immune response towards a Th1 type of response and thus enhance CTL responses to tumours (Trinchieri and Scott 1999). These effects along with the role of IL-12 in inhibition of angiogenesis, are believed to be the predominant components of the IL-12-mediated antitumour immune responses observed in mice (Lollini and Forni 1999). Clinical success of IL-12 in antitumour therapy however has been limited by the severe toxicity associated with systemic administration of this cytokine. To minimise toxic effects observed upon systemic administration and to take full advantage of the immunomodulatory activities of this cytokine, localised production of IL-12 alone or in combination with other immune potentiating agents has been exploited as a therapeutic antitumour approach with very encouraging preclinical results (Melero, Mazzolini et al. 2001) (discussed further section 1.6.3.2.1).

Recently the critical role of type I IFN on DC differentiation and function and the potent antitumour effects mediated by these cytokines have become better understood. IFN-α, the most extensively used cytokine in the clinic, can exert both direct and indirect antitumour effects. Predominantly produced by monocytes, macrophages and a specialised subtype of DC, the plasmocytoid DC (also known as IFN-producing cells), IFN-α can promote the antitumour cytotoxic effects of NK cells and macrophages (Luft, Pang et al. 1998). Importantly, it has been shown that IFN-α can provide critical differentiation and maturation signals to DC and promote their migration to lymphoid organs. IFN-α can promote B cell proliferation and antibody

production and enhance the *in vivo* proliferation and survival of memory T cells, thereby leading to an enhancement of both cellular and humoral antitumour immune responses (Belardelli and Ferrantini 2002). Most importantly, IFNs enhance antigen processing and presentation in all cells in the cytokine environment, enhancing TAA presentation for any cytotoxic T cells generated.

1.4 Tumour Escape Mechanisms

Given the complexity of cellular interactions and regulatory signals that mediate the induction and maintenance of effective antitumour immune responses, it is anything but surprising that tumours cells have developed mechanisms to evade humoral and cellular immune responses. The main mechanisms tumour cells employ to escape immune recognition will be discussed below:

1.4.1 Selection and Outgrowth of Genetically Altered Variants

Naturally arising tumours can be polyclonal in origin and tumour cells develop in such a way that antigens previously recognised by the immune system are no longer expressed. An effective antitumour immune response constitutes a selective pressure on cells and loss of expression of the immune stimulating molecules can thus lead to escape from the original immune attack. Expression of tumour antigens such as gp100, tyrosinase and MART-1/MelanA, commonly used in melanoma immunotherapy, is not homogeneous and is often significantly reduced. Suboptimal levels of antigen expression may result in inefficient T cell recognition. Selective survival advantage may also occur in tumours that possess or develop defective antigen processing and presentation machineries. Mutations in genes coding for crucial antigen presentation and transport molecules, such as TAP, and components of the proteosome, often result in selective survival of tumour cells (Maeurer, Gollin et al. 1996). So, even though the antigen and MHC molecules are present, the recognised antigenic epitope is not presented at the cell surface in the context of the MHC molecules, resulting in impaired CTL recognition and selective tumour progression (Kageshita, Hirai et al. 1999). Total or partial loss of MHC class I expression is another immune evasion mechanism employed by tumour cells (Rosenberg, Packard et al. 1988; Nawrocki and Mackiewicz 1999). Inactivation by

mutation of the β2-microglobulin gene leads to complete loss of MHC Class I expression on tumour cells. Since complete loss of MHC Class I expression on the surface of tumour cells may make them more susceptible to NK cell-mediated elimination, tumour cells may better survive with partial rather than complete loss of Class I expression.

1.4.2 Tolerance Induction

Costimulatory molecules are normally expressed by APC, such as DC, B cells and macrophages. With the exception of some lymphomas, tumour cells do not normally express costimulatory molecules and are therefore incapable of providing the crucial signal 2 for induction of an effective antitumour immune response. Interaction of TCR on $CD8^+$ T cells with MHC Class I/peptide complexes on tumour cells in the absence of costimulation leads to T cell anergy. Naïve T cells meet antigen on cells within the peripheral lymphoid organs, most commonly presented by APC. These APC may not be fully matured following contact with the tumour environment and thus express suboptimal levels of costimulatory molecules and cytokines and be tolerogenic (Blankenstein and Schuler 2002). Therefore, in the absence of acute pathological challenges, the chronic antigen source generated by the tumour generates more tolerance than immune activation despite dominant TAA. Anergy may be induced among CTL previously capable of recognition and cytotoxic function (Staveley-O'Carroll, Sotomayor et al. 1998). Anergy of $CD4^+$ T cells may lead to absence of T cell help, necessary for induction and maintenance of antigen specific CTL responses. In addition, lack of immunostimulatory or danger signals in the tumour microenvironment may lead to immune tolerance or anergy.

1.4.3 Suppression of the Immune Response

Tumour cells can secrete inhibitory factors such as tumour growth factor (TGF)-β or IL-10 that can directly or indirectly suppress immune effector cells. TGF-β blocks T cell activation while IL-10 blocks DC maturation (Torre-Amione, Beauchamp et al. 1990; Wojtowicz-Praga 1997). In addition, Fas ligand (FasL) expression on the surface of tumour cells can lead to apoptosis of T cells upon ligation of Fas on the T cell surface. FasL expression has been demonstrated in a number of malignancies

including melanoma, glioblastoma, colon carcinoma, lung carcinoma and ovarian cancer (Bremers and Parmiani 2000).

1.4.4 Impaired T Cell Function

Analysis of TIL isolated from tumours has demonstrated a general impairment of function of T cells. Downregulation or complete loss of expression of TCR components, such as CD3 ζ, leading to impaired signalling from the TCR/CD3 complex has been observed in peripheral or intratumoural T lymphocytes isolated from cancer patients. Similarly impaired $p56^{lck}$ or Zap70 tyrosine kinase function leading to impaired T cell proliferative responses has been demonstrated in TIL from cancer patients (Tartour, Latour et al. 1995). Finally, abnormalities in signal transduction pathways and transcription factors may also contribute to abnormal function of T cells. This is likely caused by the tolerance and suppression mechanisms.

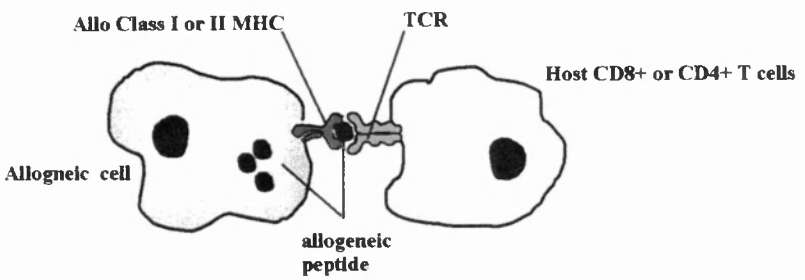
1.5 Alloresponse and Tumour Immunity

The immune system has the ability to react to allogeneic MHC molecules on transplanted cells in a highly vigorous manner. Several immunotherapeutic strategies for cancer treatment, such as adoptive transfer of allogeneic DC or allogeneic tumour cell vaccines, have tried to take advantage of this feature to compensate for weak responses to tumour antigens and to enhance specific antitumour immunity. Most of what is currently known about allogeneic responses comes from transplantation immunology and although the potential interaction between transplantation and tumour immunology has been occasionally investigated, the full potential of this interaction has yet to be vigorously explored.

T lymphocytes are the main cell type involved in tissue rejection. In a phenomenon called "alloagression", unprimed T cells from one individual have the ability to potently react against the MHC of an individual from the same species (Fabre 2001). Whereas the frequency of T cells reactive to conventional antigens is estimated to be approximately $1x10^{-5}$ to $3x10^{-5}$, the frequency of alloreactive T cells can be as high as $1x10^{-3}$ (Suchin, Langmuir et al. 2001), demonstrating the incredible potency of the

alloresponse to foreign MHC. T cells can recognise determinants on intact allogeneic MHC molecules on the surface of foreign cells in a process called "direct allorecognition" (**Figure 1.3.A**). In addition to recognising allogeneic MHC through direct allorecognition, T cells can also recognise allo MHC epitopes presented on the surface of host APC complexed to self MHC. This presentation occurs following uptake and processing via the MHC Class I and Class II pathways in a process known as "indirect allorecognition" (**Figure 1.3.B**). The exact molecular mechanisms behind direct allorecognition are still the subject of intense investigation.

A. Direct Allorecognition



B. Indirect Allorecognition

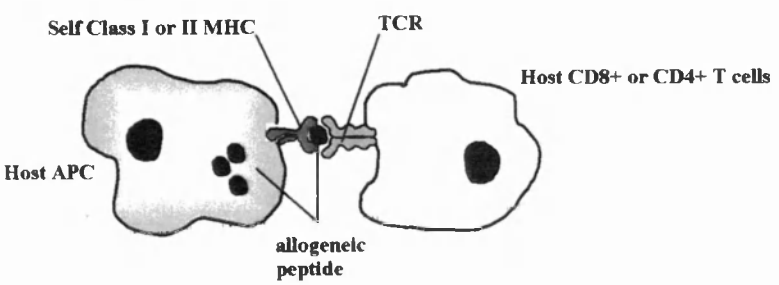


Figure 1.3: Mechanisms of allorecognition by T cells.
A: *Direct allorecogntion* involves recognition by host T cells of determinants on intact allogeneic MHC molecules on allogeneic cells. **B:** *Indirect allorecognition* involves uptake and presentation of allogeneic MHC epitopes by host APC. Host T cells recognise foreign peptide presented by self MHC molecules.

In addition to alloresponses to MHC, T cells can react to peptides from gene products of non-MHC genes. These polymorphic non-MHC proteins are called minor histocompatibility antigens (mHAgs) and T cells can only respond to mHAgs if they are presented on the cell surface by self MHC molecules. mHAgs include products of sex chromosomes, such as the H-Y antigens in mice and humans, products of autosomes, such as the HA-2 in humans, and products of mitochondrial DNA. Reactivity against mHAgs plays a significant role in transplantation rejection (Simpson and Roopenian 1997).

Tumour immunotherapy aims to generate an antigen specific systemic T cell response to tumour antigens presented by self MHC molecules, leading to the destruction of metastatic lesions. Direct T cell allorecognition has three main implications in the generation of such self MHC restricted responses (Fabre 2001). First, alloresponding T cells that can directly recognise intact allo MHC determinants may also have specificity for normal environmental peptides presented by self MHC molecules, their natural ligands (**Figure 1.4.A**). Consequently, in a cancer patient there is a random chance that alloresponding T cells also have specificity for tumour peptides presented by self MHC molecules. Second, vigorous stimulation of $CD4^+$ T cells via direct allorecognition responses to allo MHC Class II molecules can lead to very potent T cell help for existing responses to tumour peptides (**Figure 1.4.B**), overriding the need for presentation of tumour peptides by self MHC Class II molecules on APC. This may be of great significance considering that $CD4^+$ T cell help found in tumours is likely to be very weak. And third, alloresponses have the potential of generating an immunostimulatory environment at the tumour milieu, rich in cytokines and co-stimulatory molecules, leading to effective stimulation of DC, T cells and other key players composing an effective immune response.

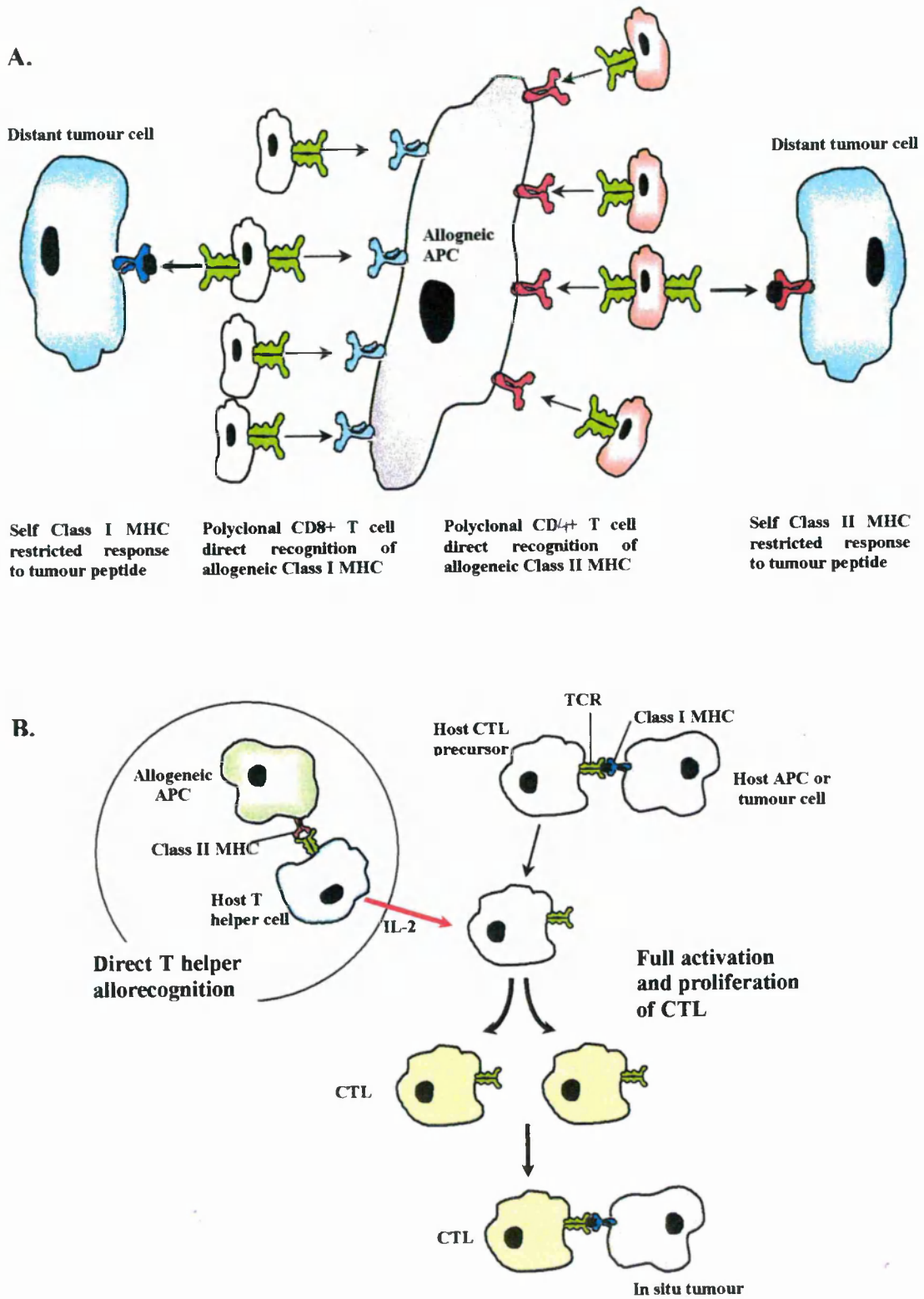


Figure 1.4: Implications of direct Allorecognition.
A: Cross-reactivity of CD4+ or CD8+ alloresponding T cells to self MHC-restricted peptides. **B:** Activated alloreactive CD4+ T cells may provide help for the generation of self-MHC restricted T cell responses to tumour peptides (modified from (Fabre, 2001)).

T cell alloagression has found some clinical application in tumour immunotherapy. One example is the adoptive transfer of antigen loaded DC. In this approach, the best suited DC for stimulating self HLA-restricted anti-tumour responses would be those that share expression of as many HLA Class I and Class II alleles with the patient but still are incompatible in at least one HLA Class II molecule. This incompatibility would ensure direct allorecognition by the patient's $CD4^+$ T cells and generation of potent T cell help promoting the induction of antigen specific HLA-specific CTL responses. Genetic modification of the patient's own DC to express an allogeneic HLA Class II allele can potentially result in the effect. Another example of the use of alloagression in tumour immunotherapy is the generation of hybrids of autologous tumour cells to allogeneic tumour cells or to DC (Gong, Nikrui et al. 2000; Kugler, Stuhler et al. 2000). Generation of "semi-allogeneic" tumour cell hybrids has had some success in preclinical studies. Recent work using human melanoma cell lines engineered to express HLA Class II and CD86 costimulatory molecules fused to tumour cells from individual patients has shown that TIL isolated from a patient and exposed to semi-allogeneic hybrids from the same patient exhibited better cytolytic activity *in vitro* (Newton, Romano et al. 2000). Subsequent use of such semi-allogeneic hybrid vaccines mixed with GM-CSF in a Phase I Clinical trial showed an enhanced antitumour response to autologous tumour cells as measured by delayed-type hypersensitivity (DTH) responses (Newton, Acierno et al. 2001). Fusion of autologous tumour cells to allogeneic or syngeneic DC has also shown great promise. In particular, fusion of autologous tumour cells to allogeneic DC represents an approach with great therapeutic potential (Gong, Nikrui et al. 2000; Kugler, Stuhler et al. 2000). The fused tumour cell/DC hybrids express all of the DC's allogeneic HLA Class II molecules stimulating $CD4^+$ T cell help via direct allorecognition, as well as all the patient specific HLA Class I for self HLA-restricted antigen specific stimulation.

1.6 Antitumour Vaccination

The underlying principle behind antitumour vaccination is the effective stimulation of the immune system against a specific component of the tumour cells, leading to immune-mediated tumour cytotoxicity and persistent immune memory. There are several prerequisite factors to stimulation of an efficient systemic antitumour

immunity. First, at least one tumour antigenic epitope against which a specific immune response can be raised need to be provided by the antitumour vaccine. Vaccines expressing multiple antigens, such as tumour cell vaccines or multivalent antigen vaccines, are better suited to minimise the risk of tumour variants not expressing a given antigen escaping immune targeting. Second, the antigens need to be released in the tumour milieu in an immunostimulatory manner. In the case of tumour cell vaccines, for example, the mechanism of cell death has been shown to influence the potency of the vaccine. Necrotic cell death of the vaccine cells, which is associated with induction and release of danger signals (Melcher, Gough et al. 1999; Basu, Binder et al. 2000), has been shown to provide an environment significantly more immunostimulatory than apoptotic cell death (Melcher, Todryk et al. 1998; Sauter, Albert et al. 2000). Third, immune cells such as DC and T cells need to be recruited at the site of vaccination for subsequent antigen uptake. Release of inflammatory cytokines and chemokines in the tumour microenvironment can efficiently promote homing and priming of immune cells. Finally, recruited immune cells need to be activated to exert their functions, for example antigen presentation and T cell activation by DC or cytokine release by cells of the innate immune system such as NK cells and macrophages. Activation signals can be provided directly by engineered vaccine cells or indirectly by appropriately activated immune cells. An effective antitumour vaccine would be one that can provide and/or facilitate all of the necessary steps for potent immune stimulation.

Advances in the molecular characterisation of TAA has allowed the development of innovative approaches to antigen-specific vaccination. Vaccination against a defined immunodominant tumour antigen provides the ability to control and target antitumour immune responses.

1.6.1 Peptide Vaccines

As previously mentioned, there is a large number of human tumours for which TAA have been identified and characterised and some of these antigens have been the focus of peptide vaccine clinical studies in cancer patients. Emerging technology has made the synthesis of large quantities of tumour antigen peptides simple, rapid and inexpensive. The use of synthetic peptides allows for safer vaccine preparations by

greatly reducing the potential for bacterial or viral contamination that is possible when using tissue for immunisation. MHC restriction of each TAA however, restrains the universal applicability of peptide vaccines. Nevertheless, peptide vaccination has been shown to be effective against viral antigens (Kast, Roux et al. 1991) and in tumour challenge experiments in pre-clinical studies (Feltkamp, Smits et al. 1993). The optimal peptide vaccine preparation should contain multiple immunodominant T and B cell epitopes, including epitopes presented by both MHC class I and class II molecules for CTL and T helper cell priming. It should contain TAA that cover a wide range of MHC types and are potent tumour rejection antigens and should be delivered by the appropriate route or vehicle such that tolerance induction is avoided, for example by providing co-stimulation or danger signals along with the TAA. Several Phase I and II clinical trial have evaluated immunisation of patients with peptides encoding epitopes of different antigens including Melan-A/Mart-1 (Jager, Ringhoffer et al. 1996; Wang, Bade et al. 1999; Jager, Hohn et al. 2002), gp100 (Rosenberg, Yang et al. 1998; Rosenberg, Yang et al. 1999; Stewart and Rosenberg 2000), NY-ESO-1 (Jager, Gnjatic et al. 2000; Gnjatic, Jager et al. 2002), and MAGE-3 (Marchand, van Baren et al. 1999; Coulie, Karanikas et al. 2002; Machiels, van Baren et al. 2002). Vaccination strategies under evaluation include direct peptide vaccination with immunologic adjuvants and/or cytokines, lipopeptide conjugates, peptide loading onto splenocytes or DC and lysosomal complexes for a wide range of cancers (Moingeon 2001; Rosenberg 2001).

1.6.2 Recombinant Viral and Bacterial Vaccines

The development of recombinant techniques that allow the insertion of antigens or antigenic epitopes into the genome of attenuated micro organisms, such as bacteria and viruses, coupled with the intrinsic immunogenicity of such agents has made the development of recombinant viral or bacterial vaccines an attractive cancer vaccine approach.

Viruses are among the most interesting vectors since they can induce antibody, as well as CTL responses. Recombinant vaccinia and other poxviruses have been the most popular vehicles used in such cancer vaccines, but more recently adenoviral and other viral vectors have also been utilized. Viral infection and the resulting cellular

pathology can elicit signals that attract and activate professional APC. Furthermore, direct infection of APC by recombinant viruses could result in efficient processing of endogenously synthesized antigens in the MHC class I pathway. Improved MHC class I and class II presentation and effective introduction of co-stimulatory molecules have been shown to enhance antigen presentation and vaccine potency (Minev, McFarland et al. 1994). Attachment of lysosomal/endosomal sorting signals to the gene encoding tumour antigen has been shown to enhance class II presentation and $CD4^+$ T cells activation by recombinant poxviruses (Lin, Guarnieri et al. 1996), and incorporation of genes for the expression of co-stimulatory proteins and cytokines into poxviruses has also been shown to enhance vaccine potency (Bronte, Tsung et al. 1995). Several Phase I/II clinical trials are ongoing for the evaluation of the feasibility of recombinant viral antitumour vaccination in melanoma, breast, prostate and lung cancers (Bremers and Parmiani 2000; Moingeon 2001; Rosenberg 2001). Exaples include vaccination of melanoma patients with adenovirus expressing gp100 or Melan-A/ Mart-1 with or without IL-2 (Rosenberg, Zhai et al. 1998), prostate cancer patients with vaccinia virus expressing PSA (Eder, Kantoff et al. 2000), and breast or lung cancer patients with vaccinia virus expressing CEA or MUC-1 (Tsang, Zaremba et al. 1995; Cole, Wilson et al. 1996; Bonnet, Tartaglia et al. 2000).

Currently a major limitation of recombinant virus vaccines is the presence of high levels of neutralising antibodies against the most commonly used viral vectors, vaccinia virus and adenovirus, in most individuals. Strategies to overcome such pre-existing immunity include engineering novel viruses which normally do not infect humans, for example viruses from the Avipoxviridae family such as fowlpox and canarypox (Bonnet, Tartaglia et al. 2000; Marshall, Hoyer et al. 2000).

The use of engineered bacteria is another interesting approach to recombinant vaccination. Bacterial strains such as *Salmonella,* BCG and *Listeria Monocytogen* possess two characteristics that make them very attractive tools for vaccination: enteric route of infection, providing the possibility for oral administration of the vaccine, and endogenous processing of the tumour antigens through direct infection of professional APC. Studies in animal models have shown promising results (Pan, Ikonomidis et al. 1995).

1.6.3 DNA Vaccines

Immunisation with DNA vaccines has gained enormous interest following the initial report by Liu and colleagues showing that intra-muscular injection of naked plasmid DNA encoding influenza nucleoprotein could protect mice from influenza challenge. Naked DNA vaccines encoding tumour antigens have been shown to provide some degree of systemic tumour protection in animal models (Irvine, Chamberlain et al. 1997). The potency of naked DNA vaccines is generally lower compared to recombinant viral vaccines. This is probably due to low *in vivo* transfection efficiencies, leading to low expression levels. In addition, inflammatory or danger responses generated by DNA inoculation are usually much weaker than those occurring during viral infections. Consequently, repeated administration of the plasmid DNA or use of adjuvant in conjunction with plasmid DNA administration is generally required for induction of an optimal response. Nonetheless, DNA injection has been shown to induce local inflammation and activation of bone marrow derived APC (Pardoll and Beckerleg 1995). The immunostimulatory activity of plasmid DNA vaccines has been associated with the presence of a central CpG motif in the sequence PuPuCpGPyPy in the prokaryotic portion of the plasmid. Through mechanisms not very well understood, unmethylated forms of such motifs can stimulate monocytes and macrophages to produce cytokines such as IL-12, TNF-α and INF-γ (Chace, Hooker et al. 1997) and enhance CTL responses and anti-tumour activity (Davila and Celis 2000). Unmethylated CpG motifs are therefore likely to play a crucial role in the ability of nucleic acid vaccines to induce immunity.

1.6.4 Tumour Cell Vaccines

Despite the significant advances that have been made in the field of TAA identification the current list of defined tumour antigens that can function as tumour regression antigens constitutes only a partial representation of the repertoire of immunologically important tumour antigens. Use of whole cells as the source of tumour antigens is a widely explored alternative approach in cancer vaccine development.

1.6.4.1 Autologous Tumour Cell Vaccines

The use of autologous tumour cells has the major advantage that the vaccine can provide the whole spectrum of unique and shared antigens expressed by the individual tumour, reducing the risk of tumour escape. First generation autologous tumour cell vaccines consisted of irradiated tumour cells isolated from fresh, cryopreserved tissue or a cell line established from autologous tissue (Greten and Jaffee 1999; Moingeon 2001). Early attempts to induce effective antitumour immune responses using irradiated autologous cell vaccines had only limited success. This apparent low efficiency could be attributed to the poor immunogenicity characteristic of most naturally evolving tumours. As mentioned above, native tumour cells often exhibit defects in antigen presentation, expression of MHC or costimulatory molecules and may directly express immunosupressive factors that can drastically limit their efficacy in inducing specific antitumour immune responses. Initial attempts to enhance the immunogenicity of autologous tumour cell vaccines employed non-specific adjuvants. Tumour cells were mixed with bacteria such as BCG and Corynebacterium parvum, components of bacterial cell walls, oil-containing mixtures such as Freund's adjuvant or aluminum salts (Greten and Jaffee 1999). Clinical trials in melanoma, colon and renal cell cancers showed enhanced efficacy (McCune, O'Donnell et al. 1990, Hoover, 1993 ; Berd, Kairys et al. 1998; Harris, Ryan et al. 2000).

One major current limitation of autologous tumour cell vaccination is that production of such customised vaccines is time consuming and technically challenging. Ex vivo expansion of tumour cells derived from the patients own tumour is cumbersome and is dependant on the quantity of available tumour tissue. This substantially limits the use of this approach in treating large groups of patients, patients with minimal residual disease, where availability of tumour tissue for *in vitro* propagation is very limited, and also limits the number of consecutive vaccinations a patient can receive.

1.6.4.2 Allogeneic Tumour Cell Vaccine

An alternative approach which eliminates the difficulties associated with generating autologous tumour cell vaccines is the use of long term *in vitro* expanded cell lines. These cell lines originate from a different patient than the patient to be treated and are therefore called allogeneic cells lines. The use of such allogeneic cell lines for

antitumour vaccination of patients gained significant popularity following the realisation that tumours from different patients, even tumours of different histological origin, often share expression of TAA and that TAA can be efficiently cross-presented by host APC to cross-prime host $CD8^+$ T cells (discussed in earlier sections). Allogeneic cell lines can be partially characterised *in vitro* for expression of known shared antigens, offer better standardisation in preparation and quality control and are amenable to large scale production and customised modification, for example genetic modification to express immunostimulatory factors. Mixture of several cell lines of the same type creates polyvalent formulations of a broad range of TAA and multiple HLA antigens, limiting the probability of immune escape. As previously discussed, expression of allogeneic HLA molecules on the surface of allogeneic cells can function as an adjuvant, rendering the cells more immunogenic as they can potentially induce both T helper and CTL immune responses to cross-reacting TAA.

Several Phase I/II clinical studies involving allogeneic tumour cell vaccines have been completed and results have triggered more elaborate Phase III studies. Morton and colleagues have generated a polyvalent allogeneic melanoma vaccine – CancerVax (Chan and Morton 1998). CancerVax is an antigen enriched melanoma cell vaccine developed from three human melanoma cell lines chosen for their rich expression of melanoma antigens. It expresses at least 11 TAA found in melanoma and other tumours and eight melanoma associated antigens. Phase II trials of CancerVax plus BCG with 157 stage IV melanoma patients (AJCC) yielded an overall response rate of 15-20% and an increased 5-year survival rate compared to historic controls (25% compared to 6%) (Morton and Barth 1996). In another Phase II clinical trial treatment of 283 stage III patients with CancerVax plus BCG yielded a 10-year overall survival rate of 49% compared to 33% in the non-vaccinated historical control patients. Randomised multicentre phase III trials with stage III and IV patients are underway to verify the effectiveness of this allogeneic vaccine to induce melanoma-specific antitumour immune responses (Chan and Morton 1998).

One variation of the theme of tumour cell vaccines is the use of tumour cell lysates or tumour-shed antigens as vaccines. Mitchell and colleagues prepared human melanoma lysates from two mechanically disrupted tumour cell lines (Melacine) and

combined this preparation with a novel adjuvant called DETOX to treat patients with metastatic melanoma in an phase I trial (Mitchell, Harel et al. 1993). Vaccination of patients with Melacine plus DETOX induced an overall objective clinical responses in 20 out of the 106 patients. Clinical responses correlated with partial match of HLA phenotype between the patients and the vaccine and increases in CTL precursors in the patient's blood. A phase III study in 140 AJCC stage IV melanoma patients however, failed to show clinical benefit from the Melacine vaccine compared to a four-drug standard chemotherapy (Mitchell 1998), although significantly lower adverse effects were associated with the Melacine vaccine.

Bystryn and co workers prepared a polyvalent melanoma antigen vaccine consisting of HLA-depleted antigens from three human and one hamster melanoma cell line (Bystryn, Jacobsen et al. 1986). In an uncontrolled, open study with 94 patients with surgically resected stage III melanoma an overall 50% 5-year survival was observed, and both cellular and humoral anti-melanoma immune responses seemed to be associated with a favourable clinical outcome (Miller, Abeles et al. 1995).

1.6.5 Enhancing Immune Response to Tumour Cells by Gene Modification

Advances in molecular biology and gene therapy techniques have allowed the cloning and characterisation of immunostimulatory molecules, such as cytokines and heat shock proteins, leading to the development of new approaches, based on genetically modified tumour cell vaccines, to enhance the immune responses to tumour cells. The principle behind cytokine gene modified tumour cell vaccines is that local sustained expression of immunostimulatory cytokines by the tumour cells will modify the microenvironmental conditions leading to induction of effective innate and/or specific antitumour immunity. Although systemic administration of cytokines such as IL-2 has been shown to lead to tumour regression, severe toxicity is associated with such an approach (Bremers and Parmiani 2000). Local release of cytokines by gene modified tumour cells therefore represents a very attractive alternative approach.

1.6.5.1 Gene-Modification With IL-12

IL-12 is a heterodimeric cytokine that exhibits proinflammatory effects, as well as antitumour properties *in vivo*. Expressed predominantly by activated DC, IL-12 promotes cellular immunity by supporting the development of Th1 responses by $CD4^+$ T cells, as well as the production of IFN-γ by NK cells, and is an enhancer of CTL and NK cell cytotoxic activity (**Figure 1.5**) (Trinchieri 1995). The antitumour and antimetastatic effects of IL-12 have been demonstrated in many mouse models (Trinchieri and Scott 1999). The observation that IL-12 deficient mice are more susceptible to chemical carcinogens and develop increased number of metastasis following injection of transplantable tumours indicates that IL-12 is an essential component of efficient tumour surveillance (Smyth, Thia et al. 2000).

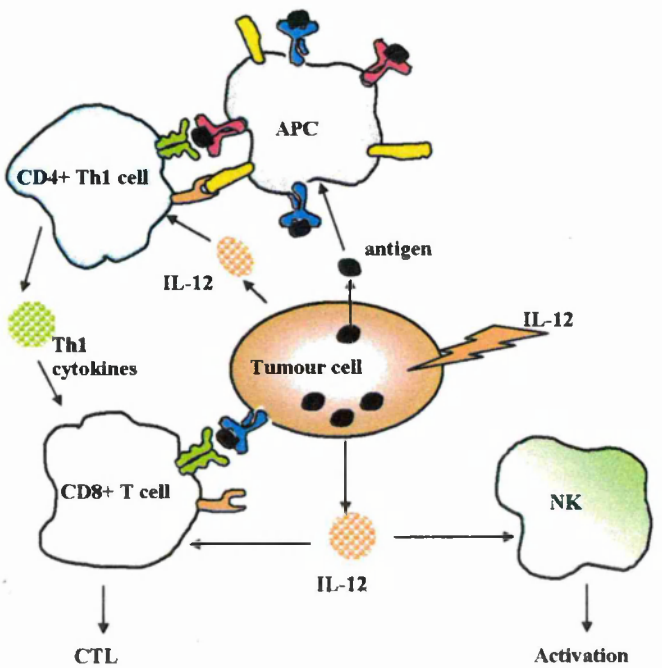


Figure 1.5: Effects of expression of IL-12 by tumour cells.
IL-12 secreted by modified tumour cells promotes a Th1- type response and stimulates the release of Th1-type cytokines by Th1 cells. In adition, IL-12 enhances cytotoxic activity of T cells and NK cells.

IL-12 has a potent direct antitumour effect, as tumour cells engineered to express IL-12 are not tumourigenic (Tahara, Zitvogel et al. 1995; Cavallo, Signorelli et al. 1997; Chong, Todryk et al. 1998) and primary tumours can be eradicated following intratumoural administration of recombinant IL-12 (Nastala, Edington et al. 1994) or recombinant adenovirus expressing IL-12 (Gambotto, Tuting et al. 1999). Furthermore, mice treated with IL-12 develop long-term tumour immunity and mice vaccinated with IL-12 secreting tumour cells or fibroblasts show enhanced systemic antitumour vaccination (Tahara, Zeh et al. 1994; Meko, Yim et al. 1995; Tahara, Zitvogel et al. 1995). The potent antitumour effect observed following gene transfer of IL-12 are dependent on T cells, particularly $CD8^+$ T cells, and IFN-γ expression by other cells, mainly NK cells. In the absence of IFN-γ, $CD4^+$ T cells and GM-CSF were required for IL-12 mediated antitumour effects (Zilocchi, Stoppacciaro et al. 1998).

Injection of IL-12-engineered fibroblasts at the site of an established MCA207 sarcoma could eliminate or suppress tumour growth in a dose-dependent manner, while repeated vaccination of animals with IL-12-secreting fibroblasts could effectively treat a pre-established sarcoma and induce long term protective antitumour immunity (Zitvogel, Tahara et al. 1995). Tumour regression was associated with infiltration of $CD4^+$ and $CD8^+$ T cells and macrophages. Similar systemic antitumour therapeutic effects were observed in mice vaccinated with tumour cells expressing IL-12 (Tahara, Zitvogel et al. 1995; Cavallo, Signorelli et al. 1997).

In addition to the direct cellular and humoral antitumour effects, IL-12 has been shown to promote the destruction of the tumour vasculature and inhibit angiogenesis (Voest, Kenyon et al. 1995; Duda, Sunamura et al. 2000; Strasly, Cavallo et al. 2001), which may contribute to its potent antitumour effects. Finally, synergistic antitumour effects have been observed when IL-12 is combined with other cytokines, such as IL-18 (Oshikawa, Shi et al. 1999) or GM-CSF (Aruga, Tanigawa et al. 1999), costimulatory molecules (Zitvogel, Robbins et al. 1996; Chong, Todryk et al. 1998) or chemokines.

Several phase I clinical trials using IL-12 gene modified tumour cell or fibroblast vaccines have demonstrated the safety and feasibility of such vaccines for cancer treatment (Sun, Jurgovsky et al. 1998; Kang, Park et al. 2001). Kang et al. (Kang, Park et al. 2001) initiated a phase I clinical study in 9 cancer patients with disseminated cancer using peritumoural injection of IL-12 gene engineered autologous fibroblasts. This was a dose escalation study where patients received four injections at intervals of 7 days. Transient reduction of tumour size was observed at the injection sites in four out of nine cases, and at noninjected distant sites in one melanoma patient. Tumour regression was associated with TNF-α expression and $CD8^+$ T cell infiltration at the local sites (Kang, Park et al. 2001).

1.6.5.2 Gene-Modification With GM-CSF

GM-CSF is one of the most extensively studied cytokines in gene modification of tumour cell vaccines. Several preclinical studies have demonstrated the potent ability of this cytokine to enhance antitumour immunity. The underlying mechanisms mediating this effect involve the augmentation of tumour-antigen presentation by recruited host APC (**Figure 1.6**). GM-CSF enhances expansion, maturation activation and migration of DC (Dranoff, Jaffee et al. 1993; Mellstedt, Fagerberg et al. 1999) leading to enhanced T cell reactivity to tumour cells as well as enhancement of primary antibody responses.

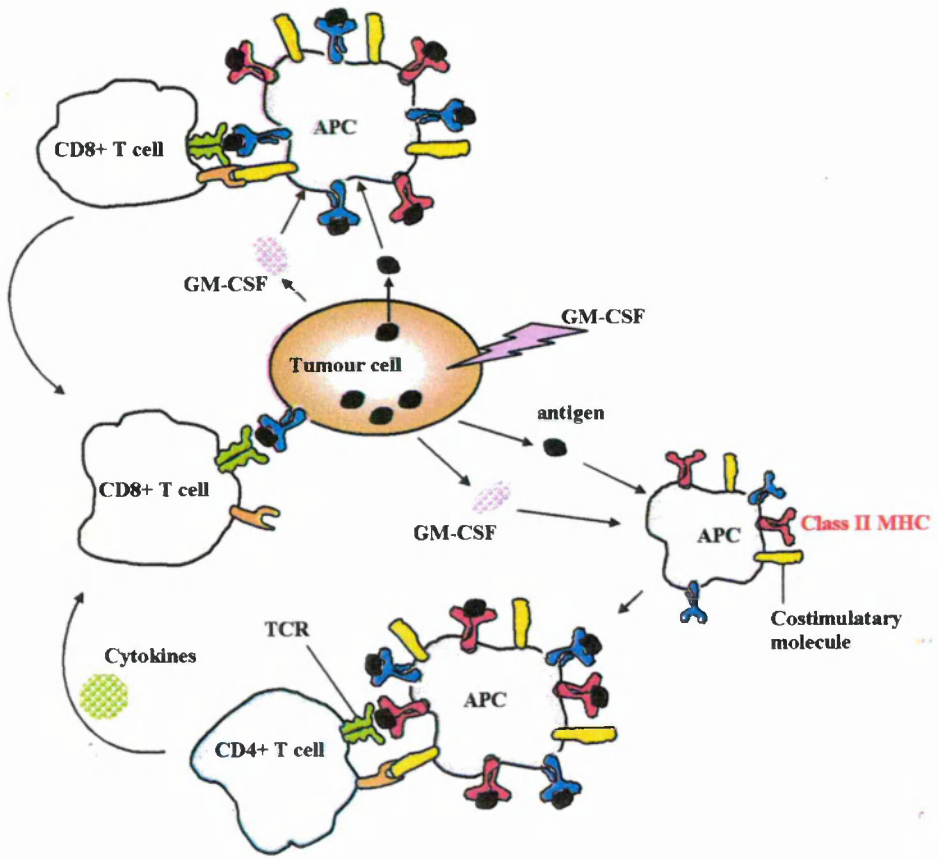


Figure 1.6: Effects of expression of GM-CSF by tumour cells.
GM-CSF secreted by modified tumour cells stimulates maturation and expansion of APC, such as DC, leading to enhanced T cell reactivity.

In a study comparing the efficacy of gene engineering of tumour cells to express different cytokines in inducing antitumour immunity, Dranoff et al. found that introduction of the gene for GM-CSF in B16 melanoma cells was superior to the insertion of other genes (Dranoff, Jaffee et al. 1993). Irradiated GM-CSF expressing tumour cells stimulated a potent, long-lasting and specific antitumour immunity against the autologous tumour. This effect was shown to be mediated by both $CD4^+$ and $CD8^+$ T cells. This study was significant as one of the first studies comparing the efficacy of irradiated gene-modified tumour cell vaccines to the efficacy of irradiated unmodified tumour cells. Further analysis of the mechanisms underlying the efficacy of such a vaccine revealed that GM-CSF expression by the vaccine tumour cells attracted a greater number of host APC which took up antigen from the dying tumour cells and cross-presented to host T cells (Dranoff, Jaffee et al. 1993; Huang, Golumbek et al. 1994; Chiodoni, Paglia et al. 1999). Expression of GM-CSF by renal cell carcinoma cells lead to complete abrogation of the tumourigenicity of primary tumours and vaccination of mice with GM-CSF-expressing tumour cells led to induction of systemic protective immunity, as demonstrated by retardation of tumour growth and prolonged survival (Kinoshita, Kono et al. 2001).

Previous work in our laboratory showed that expression of GM-CSF alone by B16 tumour cells had no effect in the growth of primary tumours while expression of herpes simplex virus-thymidine kinase (HSVtk) plus ganciclovir (GCV) alone completely abrogated growth of primary tumours (Castleden, Chong et al. 1997). When GM-CSF was combined with HSVtk/GCV treatment a significant abrogation of primary tumour growth was observed. GM-CSF release by tumour cells lead to the best levels of long-term systemic protection. Importantly, coexpression of GM-CSF with HSVtk in a tumour prevention model lead to significantly enhanced long-term protection compared to either modification alone demonstrating the ability of GM-CSF to act as a potent adjuvant in tumour immunotherapy.

The efficacy of GM-CSF modification in inducing protective systemic immunity has also been demonstrated in allogeneic tumour cell vaccination models (Kayaga, Souberbielle et al. 1999; Chang, Chen et al. 2000) with the effects dependent on both $CD4^+$ and $CD8^+$ T cells. In a study directly comparing the efficacy of cytokine modified autologous to allogeneic tumour cell vaccines, Todryk et al. showed that the

efficacy of GM-CSF transduction was significantly lower for an allogeneic vaccine compared to an autologous vaccine (Todryk, Birchall et al. 2001).

Two phase I clinical trials involving autolgous tumour cells genetically engineered to secrete GM-CSF have been reported. Soiffer et al. vaccinated patients with stage IV metastatic melanoma with GM-CSF-transfected autologous melanoma cells (Soiffer, Lynch et al. 1998). In all of the 21 evaluable patients an initially negative DTH reaction to non-transfected, autologous tumour cells was converted to a strong positive response after several vaccinations. Biopsy of metastatic lesions in 11 out of 16 patients showed dense infiltration of both T cells and plasma cells and extensive tumour destruction, and seven showed increased anti-melanoma antibody response (IgG-type). Anti-melanoma CTL and antibody responses were associated with tumour destruction, though these antitumour immune responses resulted in clinically relevant responses only in one case. Simons and co-workers conducted a similar trial with an autologous, irradiated GM-CSF expressing vaccine in renal cancer patients (Simons, Jaffee et al. 1997). In this randomised dose escalation study patients were treated with equivalent doses of autologous cells with or without GM-CSF transfer. No dose-limiting toxicity was found in the 16 evaluable patients, while an intense eosinophil infiltrate was detected at the site of DTH responses to GM vaccine but not in patients receiving non-transduced cells. One objective partial response was observed in a patient treated with GM-CSF gene transuced cells.

1.6.5.3 Gene-Modification With IFN-γ

IFN-γ is predominantly expressed by activated T cells and can significantly enhance the ability of T cells and macrophages to lyse tumour cells (**Figure 1.7**). In addition IFN-γ can activate NK cells to exert their effector functions. IFN-γ expression leads to upregulation of MHC Class II molecules in MHC Class II non-expressing cells such as epithelial cells (el-Asrar, van den Oord et al. 1989), which may lead to enhanced interaction with and activation of $CD4^+$ T cells, promoting the development of cytotoxic effector cells (**Figure 1.7**). Furthermore, IFN-γ can upregulate expression of MHC Class I molecules (Trinchieri 1994) and adhesion molecules such as ICAM-1 and LFA-3, that are important in the interaction of both MHC-restricted and non-MHC restricted effector cells with target cells. It also enhances expression of molecules involved in antigen processing and presentation including TAP and components of the proteosome complex, thereby enhancing presentation of tumour antigens (Boehm, Klamp et al. 1997).

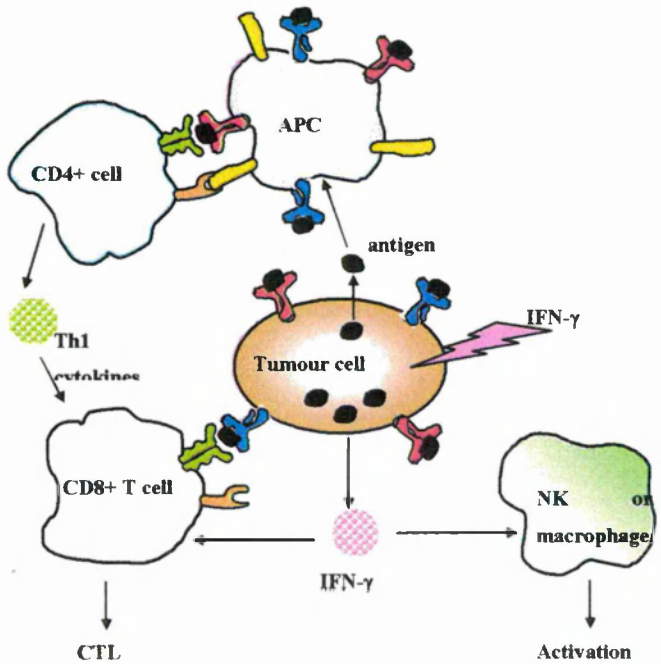


Figure 1.7: Effects of expression of IFN-γ by tumour cells.
IFN-γ secreted by modified tumour cells enhances MHC Class I and II expression and thus improves antigen presentation by cells. In addition, IFN-γ augments the cytotoxic activity of T cells as well as the effector functions of NK cells and macrophages.

Transduction of a weakly immunogenic fibrosarcoma cells line with the gene for IFN-γ significantly enhanced surface expression of MHC Class I and Class II molecules and reduced the *in vivo* growth rate of a primary tumour (Yang, Vervaert et al. 1999). In a protective vaccination model, an irradiated IFN-γ transduced tumour cell vaccine generated systemic immunity leading to significantly enhanced protection of animals against tumour challenge. These therapeutic effects were primarily dependent on $CD8^+$ T cells and partly dependent on $CD4^+$ T cells. In renal cell carcinoma, IFN-γ gene transfer enhanced tumour cell recognition by MHC-restricted, tumour antigen specific CTL but suppressed recognition by non-MHC-restricted cytotoxic cells such as LAK cells (Schendel, Falk et al. 2000). Overall, animal studies have shown that the efficacy of IFN-γ in inducing significant antitumour effects varied widely depending on the tumour model used, with some studies showing an increase in tumour metastasis following administration of an IFN-γ expressing tumour vaccine (Ferrantini and Belardelli 2000).

In a phase I clinical trial, Abdel-Wahab and colleagues treated 20 stage III-IV melanoma patients with IFN-γ secreting autologous tumour cells and demonstrated complete response in two patients and transient shrinkage of subcutaneous nodular disease in two additional patients (Abdel-Wahab, Weltz et al. 1997). Thirteen out of the twenty patients completed the protocol and eight out of those thirteen, including those that exhibited an objective clinical response, showed humoral IgG responses against autologous and allogeneic melanoma cells.

1.6.5.4 Gene-Modification With Other Cytokines

In addition to IL-12, GM-CSF and IFN-γ, several other cytokines have been explored in preclinical animal models as well as some human clinical trials for use as adjuvants in effectively inducing antitumour immunity. Local delivery of IL-2 provides a strong helper signal for $CD8^+$ T cells, possibly bypassing $CD4^+$ T helper function, leading to activation, tumour antigen recognition and enhanced tumour cell killing (Karp, Farber et al. 1993) (Russell, Eccles et al. 1991). Transfer of the gene for IL-2 into tumour cells results in inhibition of tumour growth *in vivo*, while vaccination with IL-2 expressing autologous or allogeneic tumour cells or fibroblasts has been

shown to induce an effective systemic immunity against weakly immunogenic tumours (Cavallo, Giovarelli et al. 1992; Kim, Russell et al. 1992; Kim, Russell et al. 1993; Kircheis, Kupcu et al. 2000). Tumour immunity has also been augmented by immunisation with tumour cells modified to express IL-4, IL-6, IL-7, TNF-α and IFN-α (Dranoff, Jaffee et al. 1993; Sun, Paschen et al. 1999; Kinoshita, Kono et al. 2001; Todryk, Birchall et al. 2001).

1.6.5.5 Gene-Modification With Hsp70

Heat shock proteins (HSPs) such as the cytoplasmic hsp70 and the endoplasmic reticulum-based gp96 are natural biologic adjuvants that have been explored in cancer vaccination strategies. HSPs are a highly conserved group of proteins most of which are present in abundant levels in living organisms under normal conditions (constitutively expressed HSPs). Expression of HSPs can be greatly induced by heat shock, glucose deprivation and other forms of cellular stress (inducible HSPs). Under normal physiological conditions the main functions of HSPs are binding to other cellular proteins, ensuring their correct folding and assisting in their transport to the ER and the cytoplasm. HSPs are believed to constitute potent danger signals of mammalian cell origin and thereby play a pivotal role in the development of innate and adaptive immunity (Anderson and Srivastava 2000). Accordingly, non-apoptotic (necrotic), but not apoptotic cell death, has been associated with the expression and release of high levels of immunogenic HSPs from cells (Melcher, Todryk et al. 1998; Basu, Binder et al. 2000). Release of HSPs in the extracellular milieu can lead to stimulation of macrophages and DC to secrete cytokines and upregulation of MHC and costimulatory molecule expression on DC (Suto and Srivastava 1995; Anderson and Srivastava 2000), providing a mechanisms whereby the immune system can perceive and respond to immunogenic types of cell death.

Previous work in our laboratory has demonstrated that when tumour cells are killed in a non-apoptotic manner (mediated by HSVtk plus GCV) high levels of the inducible form of hsp70 are expressed by the dying tumour cells (Melcher, Todryk et al. 1998). *In vivo*, this mechanism of cell death was associated with increased immunogenicity as demonstrated by immune protection conferred by vaccination with autologous HSVtk-killed cells against a subsequent challenge with unmodified tumour cells.

CMT93, a poorly immunogenic colorectal tumour cell line that died by HSVtk-induced apoptosis could be rendered immunogenic by overexpression of the apoptosis-blocking agent bcl-2, and this enhancement of immunogenicity was associated with increased HSP expression (Melcher, Todryk et al. 1998). Furthermore, when poorly immunogenic tumour cells were modified to overexpress hsp70 their immunogenicity could be significantly enhanced (Melcher, Todryk et al. 1998; Todryk, Melcher et al. 1999). It was therefore proposed that HSP overexpression may provide immunostimulatory signals *in vivo*, similar to those obtained by immunogenic cell death such as necrosis, which may help break tolerance to tumour antigens. These findings are consistent with those of other groups which have demonstrated that biochemically purified hsp for tumour cells can effectively stimulate a potent immune response and lead to immune protection against the parental cells (Udono and Srivastava 1994; Tamura, Peng et al. 1997; Janetzki, Blachere et al. 1998).

Although the mechanism of immunogenicity of HSPs is not completely understood, it has been suggested that endogenous heat shock proteins are not immunogenic per se but rather chaperone antigenic peptides generated during antigen processing and that it is the peptides rather that the HSPs that function as the immunogens. Evidence to support this suggestion comes from observations that HSPs stripped of their associated peptides lose their immunogenicity (Suto and Srivastava 1995). Several studies have shown that immunogenic HSP-peptide complexes can be reconstituted *in vitro* and that these reconstituted complexes are immunologically active, as demonstrated by their ability to elicit antitumour immunity and $CD8^+$ CTL responses (Blachere, Li et al. 1997). HSP-peptide complexes generated *in vivo* or *in vitro* can directly induce macrophages and CTL to secrete inflammatory cytokines such as TNF-α (Chen, Syldath et al. 1999) and can be used to effectively immunise mice against autologous tumour cells. Follow up studies in our laboratory have shown that radio-labelled lysates from tumour cells engineered to overexpress hsp70 were taken up by immature DC *in vitro* to a far greater extent than lysates of unmodified tumour cells and this effect was characteristic of immature but not mature DCs (Todryk, Melcher et al. 1999). Furthermore, experiments using tagged hsp70 demonstrated that DC specifically took up this molecule. Importantly, hsp70-expression by

tumours induced a heavy *in vivo* infiltration of T cells, macrophages and predominantly DC and expression of Th1 cytokines (Todryk, Melcher et al. 1999), an environment that is likely to promote an effective cellular immune response. These data suggest that induction of hsp70, and possibly of other HSPs, can serve to alert the immune system to an immunologically dangerous situation against which an immune reaction should be raised. These findings are in agreement with work from other laboratories that shows that HSP-peptide complexes can be taken up by APC such as macrophages, processed by the MHC Class I pathway of the APC and cross-presented on the cell surface where they can stimulate CTL (Suto and Srivastava 1995; Castellino, Boucher et al. 2000). A specific receptor, CD91, has recently been identified on the surface of APC as a common receptor for binding of several HSPs including hsp70, gp96 and hsp90 (Basu, Binder et al. 2001). The identification of this receptor may help explain the unique ability of HSPs as peptide chaperones to induce potent antigen specific $CD8^+$ T cell immune responses even in the presence of only nanomolar amounts of HSP-peptide complexes.

Collectively these observations support the use of HSPs for cancer immunotherapy. In a pilot phase I clinical study, Srivastava and colleagues treated sixteen patients with advanced malignancies refractory to established therapies with autologous tumour derived gp96 vaccines (Janetzki, Palla et al. 2000). No adverse toxicity or auto-immune reactions were associated with vaccination in any of the fourteen evaluable patients. Immunisation elicited MHC I-restricted, tumour-specific $CD8^+$ T cells in six out of twelve patients immunised, and NK cell expansion in eight of thirteen patients immunised. This study demonstrates the feasibility and safety of utilising HSPs in therapy protocols for the induction of antitumour immune responses in humans.

1.7 Fusogenic Membrane Glycoproteins

Enveloped viruses such as the orthomyxoviruses (e.g. influenza), paramyxoviruses (e.g. Sendai), rhabdoviruses (e.g. vesicular stomatitis virus), retroviruses (e.g. human immunodeficiency) and alphaviruses (e.g. Semliki Forest virus) contain the viral genome and core proteins within a membrane that has been acquired from the host cell during virus assembly and budding. Entry of enveloped viruses involves fusion of

otherwise called viral fusogenic membrane glycoproteins (FMGs). Membrane fusion is a frequent reaction in eukaryotic cells, including for example the formation and consumption of vesicles transporting molecules between intracellular organelles.

Enveloped viruses in general utilise one of two mechanisms for viral entry. Viruses belonging to the *Retroviridae, Paramyxoviridae, Herpesviridea* and *Coronaviridae* families typically initiate fusion in a pH-independent manner, where following initial attachment of the virion to the cell surface by virtue of interaction of the FMG on the virus surface with the appropriate viral receptor on the host cell surface, fusion between the viral and cellular membranes occurs at neutral pH at the cell surface (**Figure 1.8.B**) (Hernandez, Hoffman et al. 1996).

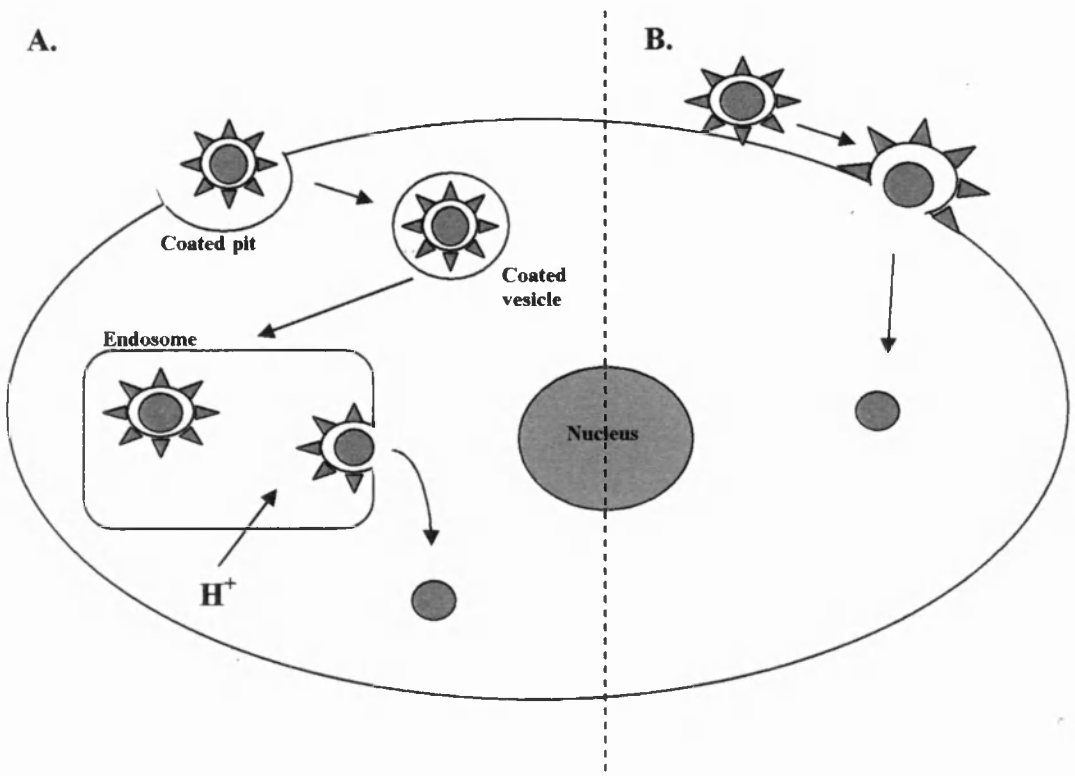


Figure 1.8: Routes of enveloped virus entry into cells.
A: Virus is endocytosed and fusion between the viral and cellular membranes takes place in a pH dependent manner in the in the endocytic compartments. **B.** Fusion between viral and cellular membranes takes place in a pH independent manner at the cell surface.

Viruses belonging to the *Rhabdoviridae, Orthomyxoviridae,* and other families typically utilise a more complex route of entry that requires a low-pH-mediated event for efficient fusion of viral and host cellular membranes (**Figure 1.8.A**). During this pathway of entry, initial attachment of the virion on the cell surface is followed by endocytosis and transport to the endosomal and lysosomal compartments, where the viral FMGs are activated by exposure to the low pH environment of these compartments. Although the exact mechanisms underlying fusion are not entirely understood, it is believed that in the pH-independent route of entry, binding of the receptor binding domain of the viral FMG is the stimulus that triggers a conformational change in the FMG molecule leading to the fusion event, whereas in the pH-dependent route, it is the lowering of the endosomal pH that triggers the conformational change.

1.7.1 Vesicular Stomatitis Virus G Glycoprotein

Vesicular stomatitis virus (VSV), a member of the *Rhabdoviridae* family of viruses, is an enveloped virus with the characteristic bullet-shaped form and contains a single stranded RNA genome of negative polarity that encodes for five viral proteins. VSV entry into the host cells involves receptor-mediated endocytosis of the viral particle into acidic endocytic organelles, where the low-pH environment of the endosomes induces fusion of the viral envelope to the cellular membrane and facilitates release of the viral core and genome in the cytoplasm (Hernandez, Hoffman et al. 1996). A single glycoprotein in the virion membrane, the G glycoprotein (VSV-G), is responsible for viral attachment and virus-cell fusion (Florkiewicz and Rose 1984; Riedel, Kondor-Koch et al. 1984). VSV-G is a transmembrane protein that exists as a non-covalently linked homotrimer on the surface of VSV particles. Glycosylation of VSV-G is critical for proper folding, assembly and transport to the cell surface (Coll 1995). It is believed that exposure to low-pH, such as that present in endosomal compartments following uptake of the virus, triggers a conformational change in the G protein allowing it to induce virus-cell or cell-cell fusion (**Figure 1.9**).

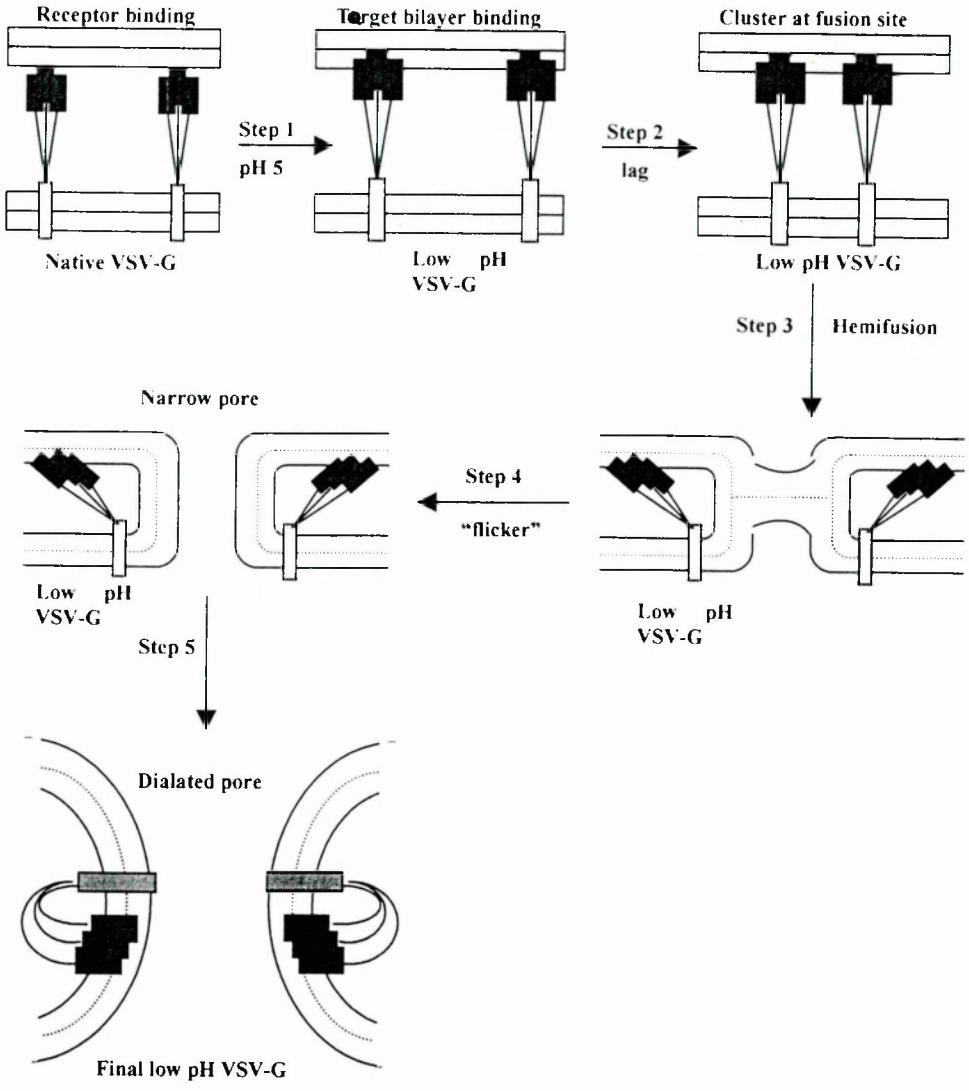


Figure 1.9: A model for VSV-G-mediated fusion.
Work on influenza HA protein has allowed the development of a model for HA-mediated fusion that can also be applied to VSV-G-mediated fusion. Conformational changes in the VSV-G protein are believed to drive the conformational transitions required to fuse two membranes (Hernandez 1996). Conformational changes of the VSV-G trimer following binding at the target lipid bilayer and clustering at the fusion site (steps 1 and 2) is proposed to promote hemifusion (step 3). During this stage of the fusion process the VSV-G trimers are thought to bend and bring the two membranes into close proximity. It is proposed that this is then followed by the formation of a small pore (step 4) and subsequent dialation of this pore (step 5) (modified from (Hernandez, 1996)).

Unlike FMGs of many other enveloped viruses the conformational change of the VSV-G has been shown to be reversible (Coll 1995). VSV-G can also initiate cell-cell membrane fusion between cells expressing the viral glycoprotein on their cell surface in the absence of any other viral proteins in a low-pH-dependent manner (Florkiewicz and Rose 1984; Riedel, Kondor-Koch et al. 1984). Cell-cell fusion leads to the formation of multinucleated syncytia (Florkiewicz and Rose 1984; Riedel, Kondor-Koch et al. 1984; Blumenthal, Bali-Puri et al. 1987). This is not however a typical cytopathic effect in the life cycle of VSV, as the G glycoprotein reaches the cell surface in a fusion incompetent form during the normal replication cycle. VSV-G-mediated fusion can be inhibited by treatment with lysomotropic agents, such as ammonium chloride and chloroquine, which accumulate in the lysosomal compartments and function by raising the lysosomal pH and thus inhibiting the conformational change in the G molecule.

Effective viral infection requires that the host cell expresses the appropriate receptor on its cell surface for VSV-G attachment and entry. Similarly, for effective cell-cell fusion to occur, a cellular receptor for the FMG must be expressed. The cellular receptor for VSV is believed to be phosphotidylserine (Schlegel, Tralka et al. 1983) (Konieczko, Whitaker-Dowling et al. 1994). Binding of phosphotidylserine to the G glycoprotein has been demonstrated in several rabdoviruses (Coll 1995). The utilisation of such a generic molecule on the surface of cells as the cellular receptor probably accounts to some extent for the extremely broad host range of rabdoviruses. It is not clear however if phosphotydilserine is the sole receptor for VSV or if it constitutes only a part of a larger protein-lipid receptor.

The G protein of VSV is a single polypeptide of approximately 500 amino acids long and is composed of a surface unit (SU), a transmembrane (TM) domain, and a hydrophilic cytoplasmic carboxy (C)-terminal peptide tail (Coll 1995). The SU contains two putative glycosylation amino acid consensus sequences and a short stretch of hydrohpobic amino acids called the signal peptide responsible for directing the nascent envelope glycoprotein through the secretory pathway of the infected cell. Near the C-terminal domain is another hydrophobic stretch of amino acids that stops the translocation process, anchors the molecule in the membrane and causes it to span the lipid bilayer (anchoring domain). Unlike FMGs of other enveloped viruses, such

as the hemagglutinin (HA) of influenza virus or the envelope of murine leukemia virus (MLV), VSV-G reaching the cell surface is an intact molecule that does not undergo proteolytic cleavage into separate subunits during transport from the ER to the cell surface (Coll 1995). For such noncleaved fusion proteins identification of the fusion domain, a peptide believed to be responsible for mediating fusion between different membranes, has been more challenging. Typically the fusion peptide is a peptide segment composed of hydrophobic amino acids that is usually located near or at the TM unit. Initial examination of the primary amino acid sequence of the G protein of VSV of different strains did not reveal the presence of any obvious hydrophobic sequence other than the signal sequence and the carboxy-terminal membrane anchoring domain. Subsequent insertional and site-directed mutagenesis studies revealed that a region of uncharged amino acids between amino acids 117 to 137 may represent a putative internal fusion peptide of VSV-G (Zhang and Ghosh 1994) (Whitt, Zagouras et al. 1990). This region is conserved between VSV serotypes.

To further support this hypothesis and to identify specific regions within this potential fusion peptide, Whitt and colleagues generated mutant VSV-G molecules by performing site directed mutagenesis and studied the folding, assembly, surface expression and fusion activity of these proteins (Fredericksen and Whitt 1995). These studies revealed at least two mutant VSV-G proteins with significantly altered fusion activities. The pH threshold for fusion activity of the wild-type VSV-G is approximately 6.3 and extensive syncytia formation in cells expressing the wild type VSV-G occurs after treatment with pH 5.7. Both VSV-G G-124→ E, which has an acidic glutamic acid instead of the non-polar glycine at position 124, and VSV-G P-127→D, which has an acidic aspartic acid instead of a non-polar proline at position 127, were fusion defective at pH 5.7 and had a greatly reduced pH threshold for fusion activity, with significant fusion observed only at pH 5.2. The extent of fusion induced by either mutant proteins was greatly reduced compared to that induced by wild type VSV-G. It is important to mention that both of the mutant VSV-G proteins were correctly folded, assembled and transported to the surface similarly to the wild type VSV-G. Collectively these studies support the existence of a critical internal fusion peptide between amino acids 117 and 137 of the VSV-G glycoprotein with an important role in the low-pH-induced virus-cell or cell-cell fusion. Fusion activity of

VSV-G can also be influenced by regions distal to the fusion peptide, suggesting that the conformation of the fusion peptide may be regulated by the three-dimensional structure of the G protein obtained after low-pH exposure (Shokralla, He et al. 1998).

VSV infection can induce both CTL and Ab responses. Although the MHC Class I-restricted CTL responses to VSV are predominantly specific for the nucleoprotein, MHC Class II-restricted CTL responses could be generated in mice defective for the Class I gene. Furthermore, Th responses, capable of enhancing both CTL and Ab responses, can be induced by epitopes on VSV-G (Burkhart, Freer et al. 1994; Coll 1995).

1.7.2 Gibbon Ape Leukemia Virus Envelope Glycoprotein

Gibbon ape leukemia virus (GALV) is a C-type retrovirus that shares many similarities in genome organisation, structure and function of viral gene products with other C-type retroviruses such as murine leukemia viruses (MLV) and feline leukemia viruses (FeLV) (Hunter and Swanstrom 1990). GALV, like many other enveloped retroviruses, enters the cells in a pH-independent manner. Fusion of the virus membrane with the cell membrane occurs at the cell surface at neutral pH (**Figure 1.10**). Viral attachment and subsequent fusion is mediated by the envelope gene product of GALV.

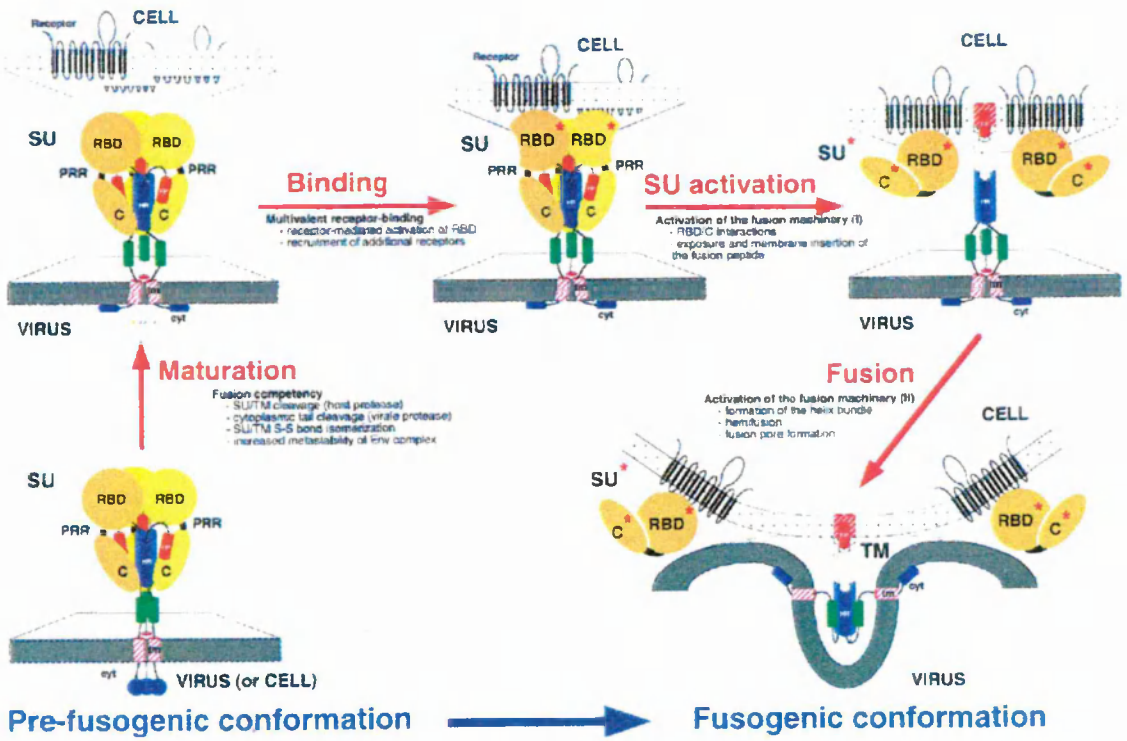


Figure 1.10: Model for the fusion process for C-type retroviruses including GALV.
A critical step for the maturation and acquisition of the fusion competent state of the envelope protein is the cleavage of the R peptide off the cytoplasmic tail. Receptor-SU binding promotes conformational changes that lead to the exposure of the fusion peptide. The fusion process following this step is similar to that proposed for VSV-G. Reproduced by kind permission of Dr D. Lavillette and Dr F-L. Cosset.

The GALV envelope shows very high homology to other C-type retrovirus envelopes such as those of MLV and FeLV and many of the characteristics of the extensively studied MLV envelope are therefore believed to be applicable to the GALV envelope. The nascent GALV envelope gene product is composed of a single 85 kDa polypeptide which is proteolytically cleaved shortly after translation into two distinct polypeptides non-covalently linked to each other. The larger subunit, which corresponds to the N-terminal region of the precursor protein, is a 70 kDa polypeptide (gp70 or surface, SU) that is completely extracellular and contains the receptor binding domain (RBD) responsible for receptor specificity and binding, a proline rich region (PRR) and a C-terminal domain. The smaller subunit, a 15 kDa polyprotein (p15E or TM) corresponding to the C-terminal region of the precursor protein, is a membrane spanning protein that plays a very important role in post-binding effects, notably fusion. TM contains an external domain, a membrane spanning domain and a cytoplasmic domain. A hydrophobic anchoring domain is contained between the membrane spanning and the cytoplasmic domain that serves to stop translocation of the polyprotein and anchor it to the membrane. TM also contains a hydrophobic region in the N-terminal region corresponding to the fusion peptide, which is important in the fusion process, and the so-called R peptide at the C-terminal end of the protein.

The R peptide is a short peptide, 16 amino acids long, which is cleaved from the TM by viral-encoded proteases during virion maturation (Sutcliffe, Shinnick et al. 1980). Cleavage of the R peptide from the TM of the envelope heterodimer is necessary to render the envelope molecule capable of triggering membrane fusion. Infection with a Moloney MLV (MoMLV) lacking the R peptide or transfection with a plasmid expressing a similarly truncated MoMLV envelope glycoprotein results in considerable cell-cell fusion (Jones and Risser 1993; Januszeski, Cannon et al. 1997; Thomas, Gray et al. 1997). The R peptide is believed to have an inhibitory role in fusion since introduction of an R peptide from another retrovirus is capable of reversing the hyperfusogenic state of a truncated envelope (Yang and Compans 1996). In addition, the R peptide is believed to play an important role in the incorporation of the envelope into the budding virions.

Shortly after translation in the ER the envelope protein is modified by N-linked glycosylation and oligomerises into trimeric structures. Oligomerisation is likely to be a prerequisite for transport from the ER to the Golgi compartment. In the Golgi the trimeric envelope precursor is further modified by trimming of the core carbohydrate side chains and cleavage by host-derived proteases into the individual SU and TM proteins. Cleavage occurs immediately after a highly conserved region of basic amino acids, placing the fusion peptide close to or at the N-terminus of the TM (Hunter and Swanstrom 1990). Following this complex series of modifications, the envelope protein is transported to the plasma membrane where it can be incorporated into assembling virions.

The cellular receptor for GALV (*Glvr* or PiT1) is a widely expressed integral membrane protein that normally functions as a sodium-dependent inorganic phosphate symporter (O'Hara, Johann et al. 1990). PiT1 has ten transmembrane regions and 5 extracellular loops. Receptor interference and superinfection resistance studies revealed that Pit1 is a receptor shared between GALV and FLV (Sommerfelt 1999). By studying differences in sequence homology between the relatively functional human Pit1 and the non-functional murine PiT1 molecules the binding sites on the receptor for GALV and FLV have been localised to the fourth extracellular loop, clustered within a region of nine amino acids termed "Region A" (Johann, van Zeijl et al. 1993). The amino acid sequence within this region was polymorphic between species, resulting in altered susceptibility to these viruses and differences in tropism between viruses. A closely related sodium-dependent phosphate symporter, Pit2, has been identified as the receptor for the amphotropic MLV (MLV-A) (Miller, Edwards et al. 1994).

1.7.3 Use of Viral FMGs As a Novel Class of Cytotoxic Genes

Several recent reports from our group and others have demonstrated that FMGs can be used as novel genes for the local control of tumour growth (Bateman, Bullough et al. 2000; Diaz, Bateman et al. 2000; Galanis, Bateman et al. 2001). Expression of a hyperfusogenic version of GALV that is lacking the R peptide, or the measles virus F and H genes, in a wide panel of human tumour lines or freshly resected human tumour explants lead to very high levels of cytotoxicity, demonstrating the great

efficacy of these genes (Bateman, Bullough et al. 2000). Similarly, expression of the VSV-G led to high levels of cell death following a drop of the ambient pH below 6.0. Direct comparison of FMG-mediated killing to the classical HSVtk/GCV- or cytosine deaminase (CD)-mediated killing demonstrated that direct and bystander killing of the three different FMGs were at least one log more potent than that of HSVtk or CD suicide genes (Bateman, Bullough et al. 2000). FMG expression led to the formation of large multinucleated syncytia, which subsequently died by sequestration of cell nuclei and nuclear fusion in a non-apoptotic mechanism. Syncytia remain stable and metabolically and transcriptionally active for a period of approximately 2 days and then lose viability by day 5 (Bateman, Bullough et al. 2000; Higuchi, Bronk et al. 2000). Expression of the hyperfusogenic GALV FMG in hepatoma cells showed that syncytial death is associated with a bioenergetic form of cell death involving necrosis and mitochondrial failure with ATP depletion (Higuchi, Bronk et al. 2000). Hyperfusogenic GALV FMG expression in human tumour cell lines was also associated with upregulation of expression of immunostimulatory signals such as hsp70 and gp96, while specific antitumour immunity could be generated in immunocompetent mice by vaccination with GALV-expressing tumour cells (GALV expression in murine cells does not lead to syncytia death as murine cells lack the appropriate Pit1 receptor) (Bateman, Bullough et al. 2000). Finally, transduction of human tumour xenografts with plasmid DNA encoding the hyperfusogenic GALV cDNA lead to significant prevention of tumour outgrowth, and cytotoxicity could be controlled using transcriptional targeting through a tissue specific promoter (Bateman, Bullough et al. 2000).

In a similar study Galanis et al. showed that expression of FMGs such as the hyperfusogenic GALV and the measles virus F and H proteins could lead to significant levels of cell killing both *in vitro* and *in vivo* in gliomas (Galanis, Bateman et al. 2001). Overall, these studies demonstrate the great potential FMGs have as potent cytoreductive agents for the local and possibly the immune-mediated control of tumour growth.

1.8 Transcriptional Control of Gene Expression for Cancer Gene Therapy

The development of gene therapy approaches for cancer treatment, particularly cytotoxic gene therapy approaches, has clearly indicated the need for the development of strategies that would maximise the beneficial effects of a given treatment and minimise the potential side effect or adverse effects. Where gene expression products are directly or indirectly toxic to the cells, restricted and highly controllable expression of the therapeutic genes to the target tissue of interest would be desired to avoid severe toxicity associated with expression in normal tissues. Control of gene expression at the level of gene transcription is one of the most widely studied approaches to targeted gene therapy, and has multiple facets due to the wide variety of promoters and transcription regulation factors that have been characterised. For the purposes of this Introduction several different promoter systems will be discussed with particular emphasis on drug-inducible promoter systems.

1.8.1 Tissue Specific Promoters

Certain tissues express proteins which are to a large degree specific to that tissue, for example tyrosinase in melanocytes and PSA in prostate cells. The promoters that regulate these genes are known as tissue specific promoters (TSP) and have been greatly exploited for the use in targeted gene expression. In the context of cancer treatment, an ideal TSP should meet the following criteria: (1) its activity should be reliably restricted to the target tissue; and (2) the normal tissue (in which it will also be active) should either be expendable, replaceable or located far from the site of gene delivery/expression. For example, a gene therapy strategy using a TSP driving a gene that ablates melanocytes, prostate or thyroid tissue is tolerable, whereas a similar approach that caused significant damage to liver or neuronal tissues would have potentially disastrous consequences. In addition, the degree of tissue specificity of a TSP is of considerable importance and will have a direct bearing on the therapeutic index of the gene therapy approach. Placing a therapeutic gene under the control of a TSP does not guarantee that its expression will be exclusively limited to the target tissue. Depending on the gene being used, low level background gene expression in other tissues may be associated with toxic side effects.

A considerable amount of work has concentrated on the use of TSP with preferential activity in melanocytes. Vile and Hart (Vile and Hart 1993) reported that inclusion of small elements of the 5' flanking sequences of the tyrosinase and TRP-1 genes in plasmid and retroviral constructs was sufficient to drive tissue specific expression of a *lacZ* reporter gene in a wide range of murine and human melanoma cell lines *in vitro*. In addition, tissue specific expression was seen in a melanoma, but not colorectal, tumour in vivo after direct injection of plasmid DNA (Vile and Hart 1993). In a subsequent study, tissue specific expression of cytokine genes was demonstrated under the control of the tyrosinase promoter and cells expressing these genes were shown to have reduced tumourigenicity *in vivo* (Vile and Hart 1994). However, direct intratumoural injection of plasmid DNA with cytokines expressed from the tyrosinase promoter led to gene expression but no tumour regression. Further refinement of this approach has been achieved by engineering Moloney murine leukemia virus-derived RV vectors with the tyrosinase enhancer/promoter replacing the 3' long terminal repeat viral enhancer (Vile, Diaz et al. 1995; Diaz, Eisen et al. 1998). Therapeutic studies *in vitro* and *in vivo*, using localised and metastatic melanoma models, have confirmed the ability of plasmid DNA and RV vectors containing suicide transgenes to selectively kill melanoma cells (Vile and Hart 1994; Cao, Kuriyama et al. 1999; Park, Brown et al. 1999).

Other TSP under development for cancer gene therapy strategies include the PSA promoter restricting expression in prostate, the albumin promoter restricting expression in liver and the ostecalcin promoter restricting expression in osteoblast (Harrington, Linardakis et al. 2000).

1.8.2 Tumour Specific Promoters

The dividing line between tissue and tumour specific promoters is rather blurred. Essentially, the definition of tumour specific promoter relies on the fact that certain promoters are either silent or have very low background levels in normal tissues, but are highly active in tumours. A number of promoters can be included within this rather heterogeneous group: (1) promoters that are specific for the malignant process but that show no particular tissue specificity – so-called "cancer-specific promoters"; (2) promoters of genes that encode oncofetal antigens and which have very well-

defined patterns of tissue specificity – so-called "tumour-type specific promoters"; (3) promoters responsive to pathophysiological conditions which predominate in tumour areas, for example hypoxia responsive promoters; and (4) promoters that are specific to cells of the tumour environment, such as the tumour vascular endothelium.

Classical examples of a tumour specific promoter utilised in the development of cancer gene therapy approaches include the carcinoembryonic antigen (CEA) and the alpha fetoprotein (AFP) promoters. CEA is expressed by a number of adenocarcinomas including colorectal, breast and lung cancers, while AFP is expressed by hepatocellular carcinomas and malignant testicular teratomas. The promoters of these genes have been characterised and their essential elements have been identified. A number of authors have used the CEA promoter to drive expression of either reporter or therapeutic transgenes in gastric, lung and colorectal tumour systems. In most of these studies, adenoviral vectors have been used with the CEA promoter controlling the expression of a suicide gene (HSVtk or CD). Such vectors have been shown to confer selective gene expression both *in vitro* and *in vivo* after intraperitoneal (Lan, Kanai et al. 1997) or intratumoural (Brand, Loser et al. 1998) injection. However, when compared to the highly active viral CMVI/E promoter, the CEA promoter was shown to have relatively weak transcriptional activity. A similar body of work exists for the treatment of hepatoma with AFP-regulated gene therapy systems. Again, many of the studies have focused on the use of TSP-driven suicide gene approaches (Harrington, Linardakis et al. 2000).

1.8.3 Inducible Promoters

Over the last ten years considerable progress has been made in the development of inducible systems. In these systems transcription of exogenous genes introduced into cells can be controlled by the administration of inducing or repressing agents by means of their actions on inducible transcription factors and specific promoters. A number of agents, including drugs, radiation and heat have been used to control such "gene switches", with particular progress being made in the development of drug-inducible systems. Three characteristics are necessary for the generation of the optimal inducible system: (1) *specificity*, as there should be no responsiveness to endogenous activators or interference with cellular regulatory pathways; (2)

efficiency, as there should be very low to no basal levels of gene expression in the uninduced state and high levels of expression upon induction, with rapid and efficient conversion between the two states; (3) *dose responsiveness*, allowing for adjustment of the levels of gene expression to meet the needs for the therapeutic requirements (Rossi and Blau 1998). In the case of drug inducible systems, the inducing agents/drug would ideally be a small, non-toxic, orally bioavailable and cell-permeable molecule capable of reversibly modulating the activity of the transcription factor. Initial small molecule inducible systems were based on endogenous transcription factors inducible by metals or steroids. These systems however suffered from the pleiotropic effects of the inducers leading to modification of expression of non-target host genes, as well as from interference from endogenous inducers such as other hormones. Efficient systems based largely on exogenous transcription factors have been developed and some of the most successfully applied systems will be discussed below.

1.8.3.1 Tetracycline Inducible Allosteric Off System

The first widely used drug-inducible system to be discussed is an entirely prokaryotic allosteric system described by Gossen and Bujard (Gossen and Bujard 1992) where gene expression is suppressed by the addition of the non-toxic antibiotic tetracycline (tet). In this system a constitutively expressed DNA binding moiety, the tetracycline controlled transactivator (tTA), consisting of the *E. coli* tet repressor (tetR) fused to the activating domain of virion protein 16 (VP16) of HSV, binds to an engineered tet responsive element (TRE) placed upstream of the TATA box of target genes and activates transcription. Addition of tet, or its more efficient analogues such as doxycycline (Dox), leads to a conformational change in the *tet*R DNA binding domain of the tTA such that it dissociates from the promoter element leading to abrogation of gene expression (**Figure 1.11.A**). The TRE is composed of seven tandem repeats of tetR binding sites and is located just upstream of a minimal CMV promoter that lacks the strong enhancer elements normally associated with the CMV immediately early promoter. In the initial study performed in HeLa cells stably expressing tTA, expression of luciferase (luc) in the presence of ng/ml quantities of tet were very low and withdrawal of tet resulted in induction ratios of as much as 100 000-fold (Gossen and Bujard 1992). Moreover, luc levels could be varied by titrating

the concentration of tet in the growth media and maximal, steady-state levels of activity were achieved in about 24 hrs. Subsequent studies however have failed to reproduce those initial impressive induction ratios, mainly due to high basal levels of gene expression. Nonetheless, the allosteric tet-off system has found very wide application in tissue culture as well as transgenic mouse studies.

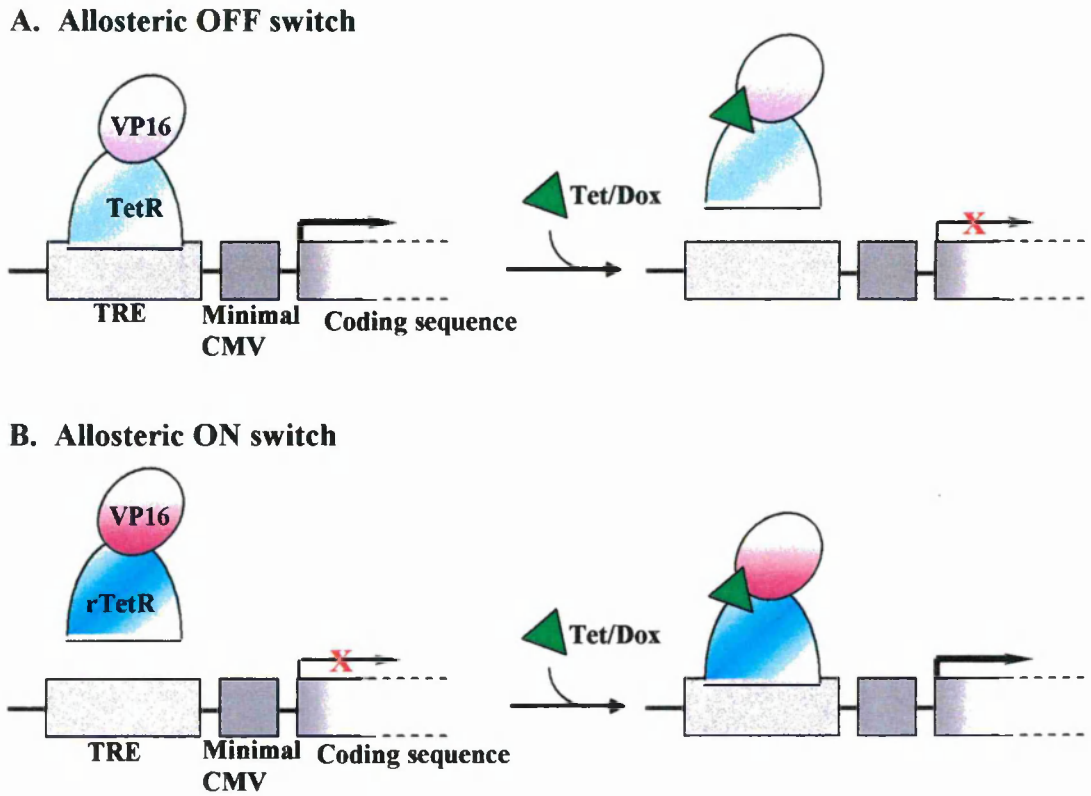


Figure 1.11: Diagramatic representation of the Tet inducible systems.
A: The allosteric Off system in which transcription proceeds in the absence of the regulating drug, tetracycline (Tet) or analoques such as doxocycline (Dox). **B:** The allosteric ON system in which transcription occurs in the presence of the Tet or Dox .

Hwang et al. developed a retroviral vector in which HSVtk was placed under the control of a tet-responsive expression system (Hwang, Scuric et al. 1996). In the absence of tet, the tk enzyme levels were 400-fold greater than in control cells but these levels returned to normal after addition of tet. Yu et al. placed the gene for IL-1β-converting enzyme, which forms part of the apoptosis signalling pathway, under the control of the tet-off system in a RV coexpressing the tTA (Yu, Sena-Esteves et al.

1996). Introduction of this retroviral vector in glioma cells led to complete death of the cells *in vitro* or when implanted into rat brain, within 4-9 days of removal of tet. In contrast, Baasner et al. expressed HER2/c-erbB2 in fibroblasts under the control of tet/tTA and showed that tumour formation could be reversibly induced *in vitro* and *in vivo* by administration of Dox (Baasner, von Melchner et al. 1996). The basic tTA system has also been used to generate transgenic mice reversibly expressing luciferase or β-galactosidase in a variety of fetal and adult tissues (Shockett, Difilippantonio et al. 1995).

The allosteric tet off system has served as a valuable model system but it has a number of potential disadvantages for clinical application. Chief amongst these is the fact that steady state levels of the modulating agent are required to keep the therapeutic gene inactive. This is inconvenient but also exposes the patient to the potential toxicities of prolonged drug administration and unplanned gene expression after inadvertent drug withdrawal. An additional problem is presented by the fact that the time course of gene activation is dependent upon pharmacokinetic parameters such as metabolism and excretion which dictate the rate of drug clearance.

1.8.3.2 Tetracycline Inducible Allosteric On System

Subsequent to the development of the original tet-off system, Gossen et al. described a modified system in which a reverse transactivator (rtTA) could bind TRE efficiently only in the presence of the tet analogues, Dox or anhydrotetracycline (**Figure 1.11.B**) (Gossen, Freundlieb et al. 1995). rtTA contains a mutated TetR that has four amino acid exchanges at positions 71, 95, 101 and 102 leading to its reversed binding properties and phenotype. Transfection of plasmids containing luciferase or β-galactosidase under the control of the tTA-dependent promoter in HeLa cells stably expressing the rtTA demonstrated the expression of the reporter genes could be induced more than 1000-fold upon administration of 1μg/ml Dox. Luciferase expression could be induced 100-fold after 5.5 hrs and reached maximal levels in less than 24 hrs. One of the main advantages this modified system has over the original tTA system is that the kinetics of induction of gene expression do not depend on the biological half-life of the effector drug, leading to much more rapid activation of gene

expression. In addition this system is particularly attractive for use in situations where repression of gene expression is desired for long periods of time and long term exposure to tet or its derivatives is undesirable or inconvenient, for example in transgenic animals or long-term gene therapy approaches).

Several groups have described technical improvements to the original tet-off and tet-on systems. To overcome technical difficulties associated with the generation of stable cell lines expressing both components of the system (tTA or rtTA plus the gene under the control of the tTA-responsive promoter) and to make the cloning process more rapid and efficient, several groups have developed single vectors containing both components for the simultaneous modification of cells. Plasmid, lentiviral, adenoviral, retroviral and adeno-associated viral vectors have been explored in this strategy (Hofmann, Nolan et al. 1996; Paulus, Baur et al. 1996; Bohl, Salvetti et al. 1998; Huang, Spinella et al. 1999; Rubinchik, Ding et al. 2000; Vigna, Cavalieri et al. 2002). To incorporate tissue specificity in the tet system several studies have developed strategies for the tissue–specific expression of the transactivators directed by tissue-specific promoters. Examples include the cardiac-specific expression of tTA and subsequently luciferase by utilising the rat α myosin promoter in rats and mice (Fishman, Kaplan et al. 1994; Passman and Fishman 1994) and pancreatic β cell restricted expression of SV40 large T antigen achieved using the rat insulin promoter (Efrat, Fusco-DeMane et al. 1995). Furthermore, vectors containing two minimal promoters in opposite orientations on either side of the TRE have been developed that allow tet-regulated expression of two genes in equal amounts from a single vector (Baron, Freundlieb et al. 1995). In an effort to activate higher levels of gene expression and to prevent possible toxic effects of constitutive expression of tTA, Shockett et al. placed tTA under the control of a tet-responsive promoter, resulting in an autoregulatory inducible gene expression system in which tTA is autoactivated in the absence of tet and expression of tTA is suppressed in the presence of tet (Shockett, Difilippantonio et al. 1995). Using this system substantially higher levels of target gene expression could be achieved *in vitro*, as well as higher frequency of resulting inducible clones. In addition, transgenic mice were produced in which expression of luciferase was 1-2 orders of magnitude higher than those reported with

the constitutive transgenic system (Furth, St Onge et al. 1994), although the levels in the uninduced state were also greater.

An important addition to the tet-on regulation system has been the incorporation of tet controlled transcriptional silencers (tTS), which are fusions between a TetR variant and domains known to function as repressors of transcription. Freundlied et al. tested several such domains and found the KRAB domain deriving from the human kidney protein Kid-1 to be the most efficient (Freundlieb, Schirra-Muller et al. 1999). The resulting TetR-fusion tTS^{Kid} can be coexpressed with rtTA and in the absence of Dox tTS^{Kid} can bind to the TRE and actively repress any basal activity. Addition of Dox leads to conformational change in the tTS^{Kid} releasing it from the TRE, while at the same time rtTA is activated to bind TRE and strongly activate transcription from the promoter. Heterodimer formation between the tTS^{Kid} and rtTA molecules is avoided due to modification of the dimerisation surface of the TetR moiety of the tTS^{Kid} that allows the tTS^{Kid} monomers to only bind to each other. This modification results in significantly lowered background levels of gene expression in the absence of Dox and hence significantly enhanced induction ratios upon Dox administration.

It is important to note that the efficacy of the tet inducible systems has varied widely between cells or tissues of different type. Since the properties of minimal promoters are critical for low background expression and high induction it is generally proposed that the success in any given cell or tissue milieu might require alternative minimal promoters and careful choice of constitutive or tissue-specific promoters for transactivator expression (Shockett and Schatz 1996).

1.8.3.3 Dimerisation On Switch

An alternative to the allosteric switch systems is provided by strategies based on drug-induced dimerisation of separate DNA-binding and activating proteins. Such systems using immunosuppressive agents such as cyclosporine A, FK506 and rapamycin that bind to members of the immunophilin protein family have been extensively investigated. Initially, in a manner analogous to that described above for tetracycline-regulated systems, two chimeric fusion proteins were constructed (one a fusion between a Gal4 DNA-binding domain and FKBP12, the other between cyclophilin

and the VP16 transactivator) (Belshaw, Ho et al. 1996). In this study, addition of a cyclosporine-FK506 fusion compound strongly promoted heterodimerisation between these two fusion proteins and subsequent transcriptional activation. Attempts have recently been made to improve this system by substituting the non-eukaryotic elements with mammalian equivalents in an attempt to reduce the potential for immune reactions. Therefore, Gal4 has been replaced by the ZFHD1 DNA binding domain and VP16 has been replaced by the p65 subunit of NFkB. A further modification involved replacing the cyclophilin element with the FKBP-rapamycin binding (FRB) domain from the FKBP12-rapamycin associated protein (FRAP) (Magari, Rivera et al. 1997). With this system, rapamycin is able to control the expression of transgenes *in vivo* in a predictable fashion. However, the immunosuppressive effects of the modulating compound has been seen as a significant potential disadvantage to clinical application of this system. Therefore, rapamycin analogues (so-called rapalogs) have been identified which do not interact efficiently with wild-type FRAP but which are capable of binding to mutant FRB proteins. The rapamycin has been shown to yield regulated expression of human growth hormone in nude mice (Rivera, Clackson et al. 1996; Rivera, Ye et al. 1999), and regulated production of cytotoxic genes and gene therapy vectors for cancer therapy (Chong, Ruchatz et al. 2002).

1.8.3.4 *Customised Promoter*

The perceived limitations of many of the natural promoter systems have fuelled the search for means of improving available wild-type sequences to derive so-called customised or designer promoters (Nettelbeck, Jerome et al. 2000). The simplest of these strategies involves the attempt to identify mutant promoters with increased ability to drive transcription but with preserved tissue/tumour specificity (Ishikawa, Nakata et al. 1999). More benefit has accrued from attempts to generate minimal enhancer/promoter sequences that retain the characteristics of the wild-type elements. Such studies involve generating a range of deletion and amplification mutants of the enhancer/promoter sequence driving a convenient reporter gene such as lacZ or chloramphenicol acetyltransferase (CAT). The DNA sequences that are derived from these studies are invariably smaller than the wild-type sequence, a feature that is of considerable significance to the issue of generating viral vectors. Such studies have

identified the crucial transcriptional control sequences for a number of genes and have led to the construction of a number of new smaller modular versions of previously identified promoters. Another strategy involves the construction of chimeric promoters composed of regulatory elements from different promoters with the same tissue specificity. This tactic seeks to exploit the advantages of each of these promoters and to tighten the tissue specificity. This has been applied for the AFP and albumin promoters with some success in a hepatoma models (Su, Chang et al. 1996; Su, Lu et al. 1997). Alternatively, TSP have been used to drive the expression of exogenous bacterial or viral transcription factors which can subsequently transactivate constructs containing therapeutic genes downstream of the appropriate DNA binding sites (Segawa, Takebayashi et al. 1998). It is likely that further developments will occur rapidly in this exciting arena.

1.9 Aims of the Thesis

Work presented in this thesis focused in the design of effective allogeneic vaccines for enhancing the immune responses against tumour cells. The first part of the thesis will investigate genetic modification of allogeneic tumour cells with genes expressing cytokines (specifically IL-12, GM-CSF and IFN-γ) or hsp70 as a means of transforming a poorly immunogenic allogeneic melanoma vaccine to a potently immunogenic one. The aim is to test whether expression of immunogenic cytokines by allogeneic cells can modify the vaccine microenvironment and thus significantly augment specific anti-tumour immune responses. This will be followed by studies aiming at understanding the mechanisms by which the immune system responds to allogeneic cells (chapters 3 and 4).

In the second part of the thesis, a novel class of cytotoxic genes, the viral FMGs, will be investigated as a novel means of inducing fusion and subsequently killing vaccine cells in a highly immunostimulatory fashion (chapter 5). Following on from previous work in the laboratory, the immunostimulatory mode of syncytial cell death, combined with the immunogenic component of allogeneneic cells is tested as a potent inducer of specific anti-tumour immune responses. The mechanisms of immunogenicity of FMG induced cell death will also be investigated (chapter 6).

Finally, the third part of the thesis will involve the development of strategies for the control of FMG expression for the purpose of generating "off-the-shelf" fusing allogeneic vaccines. The Tet-On drug-inducible gene expression system will be utilised for the development of human allogeneic melanoma cell lines stably expressing the GALV envelope gene. Such cell lines can find significant clinical application as they can form the basis of an allogeneic fusing tumour cell vaccine for use in human clinical studies.

Chapter 2

MATERIALS AND METHODS

2.1 Molecular Biology

2.1.1 General Procedures

All solutions employed for the preparation and manipulation of nucleic acids were made up using distilled water (dH_2O). All solutions were autoclaved before use or, in the case of thermolabile substances, filter-sterilised using a 0.2 μm filter and stored in a sterile container. Unless stated otherwise, all chemical reagents were supplied by Sigma (St. Louis, MO) and all enzymes used were purchased from New England BioLabs (Beverly, MA). To reduce the chances of RNA degradation by contaminating RNAses, all solutions used for RNA work were treated overnight with diethylpyrocarbonate (DEPC) as a 0.1% v/v solution and then autoclaved.

4.1.2 Concentration of nucleic acids

Solutions containing nucleic acids were adjusted to 0.3M with respect to monovalent cations by addition of 3M sodium acetate (pH 5.0) stock solution. Nucleic acids were precipitated by adding 2.5 volumes of ethanol and chilled at -70°C for 30 minutes followed by centrifugation at 12,000*g* for 20 minutes. The pelleted nucleic acid was then washed with 70% v/v ethanol and then air dried before resuspension in distilled water or Tris-EDTA buffer (TE buffer: 10 mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0).

2.1.3 Determination of nucleic acid concentrations

The absorbance of an aqueous solution of the nucleic acid was measured at 260 nm (UV–1601, Spectrophotometer, Shimadzu Corporation, Kyoto, Japan). The convention was used that an absorbance of one unit is equivalent to a double stranded DNA concentration of 50μg/ml and an RNA concentration of 40μg/ml.

2.1.4 Transformation of bacteria

The plasmid DNA was added to 100µl of competent *E.coli.* The suspension was cooled on ice for 45 minutes, warmed at 42°C for 1 minute and then returned to ice for 2 minutes. 400µl of L-broth was then added to the samples followed by incubation in a shaking incubator at 37°C for 60 min to permit expression of the antibiotic resistance gene on the plasmid. The bacteria were then plated out onto 90mm petri dishes (Becton Dickenson Labware, NJ) containing L-agar (L-broth with 1.5% w/v agar) with ampicillin (final concentration of 100µg/ml). The plates were incubated overnight at 37°C.

2.1.5 Small scale preparation of plasmid DNA ("miniprep")

Plasmid DNA was prepared from small cultures of bacteria using a QIAprep Spin plasmid minipreparation kit (Qiagen, Valencia, CA), following the protocol supplied by the manufacturer. This procedure was based on the alkaline lysis method for rapid extraction of plasmid DNA from bacterial cells followed by the adsorption of DNA onto silica in the presence of high salt.

Single bacterial colonies were inoculated into 5 ml of L-broth containing ampicillin and incubated overnight in a shaking incubator at 37°C. 1.4 ml of the overnight cultures were centrifuged at 10,000*g* for 5 min and the bacteria were then resuspended in 250 µl of resuspension buffer P1 (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 100 µg/ml RNAse). 250 µl of lysis buffer P2 (200 mM NaOH, 1% SDS) was then added and mixed, followed by adding 350 µl of neutralisation buffer N3 which adjusts the sample to high salt binding conditions and causes precipitation of denatured proteins, SDS, cellular debris and chromosomal DNA. The samples were then centrifuged at 10,000*g* for 10 min and the supernatants were then applied QIAprep spin column and centrifuged for 30-60 sec for the samples to flow through the silica membrane which forms the floor of the spin column. After washing with 0.5 ml of buffer PB to remove trace nuclease activity and 0.75 ml of buffer PE to remove salts, the samples were centrifuged for an additional 1 min to remove any residual wash buffer and the DNA was eluted by applying 50 µl of distilled water to the silica membrane and centrifuging for 1 min.

2.1.6 Large scale preparation of plasmid DNA ("maxiprep")

Qiagen Plasmid Maxi kit was used which is based on the modified alkaline procedure followed by binding of plasmid DNA to an anion-exchange resin. A single bacterial colony was used to inoculate a 2 ml volume of L-broth containing ampicillin which was incubated for 8 hrs in a shaking incubator at 37°C. 1ml of this culture was used to inoculate 100 ml of L-broth containing ampicillin which was then incubated overnight. The bacteria was pelleted by centrifugation at 6,000*g* for 20 min (J2-HS centrifuge, Beckman) and resuspended in 10 ml of resuspension buffer P1. 10 ml of lysis buffer P2 was then added and left at room temperature for 5 min. 10 ml of neutralisation buffer P3 (3M potassium acetate pH 5.5) (pre-chilled to 4°C) was added and the lysate poured into a QIAfilter Maxi cartridge and incubated at room temperature for 10 min. The cell lysate was then filtered onto a QIAGEN-tip which had been pre-equilibrated with 10 ml buffer QBT (750 mM NaCl, 50 mM MOPS pH7.0, 15% ethanol, 0.15% Triton X-100) and allowed to enter the anion-exchange resin by gravity flow. Under these conditions, the plasmid DNA binds to the anion-exchange resin. The resin was then washed with 60 ml of medium-salt buffer QC (1 M NaCl, 50 mM MOPS, pH 7.0, 15% ethanol) to remove RNA, proteins and low molecular weight impurities. The DNA was eluted with 15 ml of high salt buffer QF (1.25 M NaCl, 50 mM Tris-HCl pH 8.5, 15% ethanol) and was then desalted by precipitation with 10.5 ml isopropanol. The DNA was pelleted by centrifugation at 15,000*g* for 30 min at 4°C ,washed with 70% v/v ethanol, air dried and then dissolved in TE buffer.

2.1.7 Agarose gel electrophoresis of DNA

Gels were prepared by adding agarose (0.7 to 1.5% w/v) to 150 ml 1 x TAE (Tris-acetate-EDTA) buffer (diluted from 50X TAE stock solution: 2 M Tris base, 2 M glacial acetic acid, 50 mM EDTA) and boiled in a microwave cooker for 5 min. On cooling to below 50°C, 2 µl of ethidium bromide stock solution (10 mg/ml) was added. Gels were poured into a gel former with a well-comb in place. After setting, the gel was submerged in an electropheresis tank containing 1 x TAE buffer. Loading buffer (1/6 volume of 6X stock solution: 0.25% bromophenol blue, 40% w/v sucrose

in dH_2O) was added to the DNA solutions which were then transferred into the wells, and electropheresis was performed using a voltage between 70 and 110 volts. The gel was transilluminated with short wave ultraviolet light and the DNA was visualised by 2 UV transilluminator (UVP, Upland, CA) and Alpha Ease 5.04 Software (Alpha Innotech Corporation, San Leandro, CA). DNA fragments were sized by reference to a 1 kb DNA ladder (New England BioLabs, Beverly, MA) or a 100 bp DNA ladder (New England BioLabs, Beverly, MA).

2.1.8 Digestion of DNA with restriction enzymes

Plasmid DNA was digested in volumes of 30 µl using 1-2 units of enzyme per µg of DNA, buffers supplied by the manufacturer and incubated for 60 min at the appropriate temperature; BSA was added when indicated.

2.1.9 Removal of 5' terminal phosphate groups

Re-ligation of the vector DNA in cases where cohesive ends were present was reduced by treatment of the restriction enzyme digestion products with calf intestinal alkaline phosphatase, to remove the 5' phosphate groups of linear double stranded. The 30 µl reaction sample from the digestion reaction was mixed with 1 unit of CIAP (Promega, Madison, WI), 5 µl of 10x reaction buffer (50 mM Tris-HCl pH 9.3, 1 mM $MgCl_2$, 0.1 mM Zn Cl_2 and 1 mM spermidine) and the reaction mixture made up to 50 µl with dH_2O. This was then incubated for a further 60 min at 37°C and the sample was run on an agarose gel and the appropriate fragment was purified as described below.

2.1.10 Purification of DNA restriction fragments

DNA purification from an agarose gel was performed using the QIAquick gel extraction kit (Qiagen, Valencia, CA) following the manufacturer's instructions. The method is based on the binding of DNA to silica under high salt conditions. Agarose gels were visualised by UV transillumination and the bands of interest excised using a scalpel blade. The excised portion of gel was dissolved in 3 volumes of buffer QG and incubated at 50°C until the gel has completely dissolved. 1 volume of

isopropanol was then added if the DNA fragment being purified was between 500-4000 base pairs and the sample was then added to the QIAquick column and centrifuged at ≥10,000*g* for 1 min. The column was then washed once with 500 µl of buffer QG and centrifuged as before. Salts were removed by washing with 750 µl of buffer PE. The DNA was eluted from the column by the addition of 30 µl TE, waiting 1 minute before recentrifugation. 1µl of the eluate was run on an agarose gel to confirm successful purification of the DNA fragment.

2.1.11 Ligation of DNA fragments into vectors

DNA fragments were ligated into vectors by overnight incubation at 14°C in the presence of T4 DNA ligase (Boerhinger Mannheim Roche, Indianapolis, IN). The molar ratio of vector to insert was in the range of 1:3 to 1:10. The appropriate volumes of DNA fragment and vector were mixed with 1 unit of T4 DNA ligase, 1.5 µl ligase buffer (50 mM Tris-HCl pH 7.8, 10 mM $MgCl_2$ 10 mM DTT, 1 mM ATP, 25 µg/ml BSA) and the reaction mixture was made up to 15 µl with dH_2O. Reaction samples were such that the concentration of the 5' termini was 0.1-1.0µM

2.1.12 Automated sequencing of DNA

Automated DNA sequencing was performed at MAYO Sequencing Core facilities using Perkin Elmer ABI Prism 377 DNA sequencer and read with Sequencher software (Gene Codes Corporation, Ann Arbor, MI).

2.1.13 Genomic DNA preparation from eukaryotic cells

Qiagen genomic DNA minipreparation kit was used kit (Qiagen, Valencia,CA), following the protocol supplied by the manufacturer. Approximately 5 x 10^6 cultured cells were pelleted by centrifugation at 1500*g* for 5 min, washed twice with 0.5 ml of phosphate buffer saline (PBS, pH7.4) and resuspended in 0.5 ml of cold PBS (4°C). Cells were lysed by adding 0.5 ml of ice cold buffer C1 (1.28 M sucrose, 40 mM Tris-Cl, pH 7.5, 20 mM $MgCl_2$, 4% Triton X-100) and 1.5 ml of ice-cold dH_2O, mixing and incubating on ice for 10 min. The lysate was then centrifuged at 1300*g* at 4°C for 15 min and the nuclear pellet washed by adding 0.25 ml of ice-cold buffer C1 and

0.75 ml of ice-cold dH_2O, vortexing to resuspend and centrifuged at 1300*g* at 4°C for 15 min. The nuclei were lysed by adding 1 ml of buffer G2 (800 mM guanidine HCl, 30 mM Tris-Cl, pH 8.0, 30 mM EDTA, pH 8.0, 5% Tween-20, 0.5% Triton X-100), containing 25 µl of QIAGEN Protease and incubating at 50°C for 60 min. Samples were vortexed thoroughly and applied on a previously equilibrated QIAGEN Genomic-tip 20/G column and allowed to enter the resine by gravity flow. Under these conditions the DNA selectively binds to the column, while degraded RNA, cellular proteins and metabolites are not retained. The DNA is then washed with 3 x 1 ml of the medium-salt buffer QC (1 M NaCl, 50 mM MOPS, pH 7.0, 15% isopropanol) to remove any remaining contaminants and eluted with 2 x 1 ml of buffer QF (1.25 M NaCl, 50 mM Tris-Cl, pH 8.5, 15% isopropanol). The eluted DNA is precipitated by mixing with 1.4 ml room-temperature isopropanol to the eluted DNA and centrifuged immediately at 6000*g* for 30 min at 4°C. The DNA pellet was washed with 1 ml of cold 70% ethanol, centrifuged at 6000*g* for 10 min at4°C, air-dried for 10 min and dissolved in TE buffer (10 mM Tris-Cl, pH 8.0, 1 mM EDTA, pH 8.0).

2.1.14 Analysis of genomic DNA by Southern blot

2.1.14.1 Preparation and electrophoresis of DNA samples

DNA samples were quantified as described previously and 5 µg were digested with the restriction enzyme *Xho*I. 10 units of enzyme were initially added in a 40 µl reaction containing 10% *Xho*I buffer and incubated at 37°C for 2 hrs. 10 more units of enzyme were added to this mixture every 2-3 hrs for 5 hrs and the reaction was incubated at 37°C overnight. The digested samples were run on a 1% (w/v) agarose gel containing ethidium bromide at 80-90 V for approximately 2 hrs as previously described. The DNA on the gel was fragmented by incubating the gel in 500 ml of 0.25 N HCl at room temperature with gentle agitation until bromophenol blue indictor turned yellow. The gel was then incubated in denaturing buffer (3 M NaCl, 0.4 M NaOH) for 30 min at room temperature with gentle agitation and then washed in transfer buffer (3 M NaCl, 8 mM NaOH) until bromophenol blue indicator turned from yellow to blue.

2.1.14.2 Capillary transfer of DNA from agarose gels

Transfer on the DNA to a nylon transfer membrane (Nytran supercharge, Schleicher and Schuell, Keene, NH) was performed overnight by downwards transfer using the TurboBlotter apparatus and Turboblotter blotter pack (Schleicher and Schuell, Keene, NH) according to the manufacturer's instructions. Following transfer the membrane was neutralised in 0.2 M sodium phosphate, pH 6.8 for 3 min and the nucleic acid immobilised on the membrane by UV cross-linking using UVC UV Crosslinker (Hoefer Pharmacia Biotech Inc, San Francisco, CA).

2.1.14.3 Generation of radio-isotope labelled DNA probe

The DNA probe was produced using (α-^{32}P)dCTP and the Prime-It II Primer Labelling kit (Stratagene, La Jolla, CA) according to manufacturer's instructions. The appropriate template DNA fragment was obtained from a suitable plasmid by restriction enzyme digest or by PCR, run on a gel and then gel extracted as previously described. This process yielded at least 250 ng of DNA fragment. 25 ng of DNA template was mixed with dH_2O to a volume of 23 µl in a microcentrifuge tube. 10 µl of random oligonucleotide primers was added and the mixture incubated at 100°C for 5 min. The tube was then centrifuged briefly and kept at room temperature. 10 µl of 5x dCTP primer buffer was added along with 5 µl (α-^{32}P)dCTP and 1µl Exo (-) Klenow enzyme. This was then incubated at 37°C for 30 min before 2 µl stop mix was added. The probe was purified by loading on to a NucTrap probe purification column (Stratagene, La Jolla, CA) and centrifuged for 10 min at 4,000 rpm. The recovered probe activity was confirmed by aliquoting 1µl into a scintillation vial containing 9 ml of Opti-fluor (Packard Instrument company, Meriden, CT) and read on a β counter LS-6000 SC (Beckman Coulter, Fullerton, CA).

2.1.14.4 Hybridisation of RNA immobilised on membrane

The membrane was placed in a hybridisation glass cylinder, 20 ml of pre-warmed (65°C) hybridisation buffer added (Rapid-hyb, Amersham Pharmacia Biotech, Pistcataway, NJ) and incubated in a hybridisation oven (Hybaid, Robbins Scientific, Sunnyvale, CA) at 65°C for 2 hrs with steady rotation. The DNA probe was heat-

denatured by boiling for 5 min and immediately placing in ice and added in pre-warmed hybridisation buffer to a final concentration of 1-5 x 10^6 cpm/ml. The hybridisation buffer in the hybridisation cylinder was replaced with the probe-containing buffer and hybridisation was allowed to proceed for 2 hrs at 65°C.

2.1.14.5 Washes and stripping of probe from membrane

The blot was washed in 100 ml of 2x SSC (20x SSC stock solution: 3 M NaCl, 0.3 M Na citrate, adjust pH to 7.0, bring to 1 L with dH_2O) at room temperature for 20 min, followed by two 20 min washes in 0.2x SSC, 0.1% (w/v) SDS at 65°C with rotation. The membrane was then placed in a plastic protector and placed against Kodak film, stored at –70°C and exposed for 1-24 hrs prior to developing.

Stripping of the membrane was performed using stripping buffer (1% (w/v) SDS, 0.1x SSC, 40 mM Tris-Cl pH 7.5 made up to 1000ml with dH_2O) heated to ~95 °C and poured over the membrane followed by gentle shaking for 10 minutes. This was repeated 3 more times. If additional stripping was required this was performed using the previously mentioned stripping buffer at 50%, with 50% formamide, heated to 65°C and poured over the membrane followed by gentle shaking for 10 minutes. Again this was then repeated 3 further times. The membrane was then able to be stored at room temperature or re-probed with a different DNA probe.

2.1.15 Total RNA preparation from cultured eukaryotic cells

RNA was obtained from adherent cell lines by employing an RNeasy Mini kit (Qiagen, Valencia,CA) which uses the selective binding properties of a silica-gel-based membrane. 1-5x10^5 cells were trypsinised and pelleted and then lysed in 350 µl buffer RLT solution (containing guanidinium isothiocyanate). The lysate was then homogenized by passing it through a 20-G needle fitted to a syringe. One volume of 70% ethanol was then added and mixed well. The mixture was then added onto a RNeasy mini spin column and centrifuged for 15 seconds at ≥8000*g*. The RNeasy column was then washed with 700 µl buffer RW1 and centrifuged as before. Next, 500 µl of buffer RPE was added to the column and centrifuged twice. After

centrifugation the RNA was eluted by addition of 30µl Rnase-free dH_2O directly onto the RNeasy membrane and centrifuged as before.

2.1.16 Total RNA preparation from frozen animal tissue

RNA was prepared from snap-frozen animal tissue using the RNeasy Mini kit (Qiagen, Valencia,CA). Frozen tumour tissue was minced using sterile scalpel blades and placed in 350 µl of lysis buffer RLT. From this point onwards the protocol was the same as described above.

Total RNA prepared for analysis with reverse-transcriptase-PCR or Northern Blot was treated with DNase I to remove chromosomal DNA contamination for the RNA. This was carried out by incubating 5 µg of RNA with 10 units of DNAse (RNAse free) (Boerhinger Mannheim Roche, Indianapolis, IN) at 37°C for one hour.

2.1.17 Quantitative analysis of mRNA by Northerm blot

The Northern blot protocol followed for analysis of RNA was similar to the Southern blot protocol previously described for DNA analysis.

2.1.17.1 Preparation and elecrophoresis of RNA samples

10 µg of DNase-treated RNA samples were made up to 20 µl with diethyl pyrocarbonate (DEPC) treated dH_2O and 2.5 µl of 5x RNA loading buffer was added (16 µl saturated bromophenol blue solution, 80 µl 0.5 M EDTA, pH 8.0, 720 µl 37% (=12.3 M) formaldehyde, 2 ml 100% glycerol, 3.084 ml formamide, 4 ml 10x 3-(N-morpholino)propanesulfonic acid (MOPS) buffer (200 mM MOPS, 50 mM sodium acetate, 10mM EDTA, pH 7.0), 100 µl DEPC water). The samples were heated to 65°C for 4 min and kept on ice until loading on a 1.2% (w/v) agarose gel (3.6 gr agarose, 30 ml 10x MOPS buffer, made up to 300 ml with DEPC dH_2O, heated in microwave oven until agarose fully dissolved, cooled to ~65°C, 2.7 ml formaldehyde and 5 µl ethidium bromide added and poured to solidify). The gel was run at 80 V for 30 min in 1x running buffer (100 ml 10x MOPS, 20 ml 37% formaldehyde, 880 ml DEPC water) to equilibrate and was loaded with the RNA samples and run at 80 V for

2-3 hrs. The gel was then imaged under UV light, washed four times with DEPC water and maintained in 20x SSC (3 M NaCl, 0.3 M Na citrate, pH 7.0, made up to 1 L with DEPC water).

2.1.17.2 Capillary transfer of RNA from agarose gels

Transfer on the RNA to a nylon transfer membrane (Nytran supercharge, Schleicher and Schuell, Keene, NH) was performed overnight by the downwards transfer method previously described using 20xSSC as the transfer buffer. Following transfer the membrane was neutralised in 2x SSC for 3 min and the nucleic acid immobilised on the membrane by UV cross-linking using UVC UV Crosslinker (Hoefer Pharmacia Biotech Inc, San Francisco, CA).

2.1.17.3 Generation of radio-isotope labelled DNA probe

Same as section *2.1.14.3.*

2.1.17.4 Hybridisation of RNA immobilised on membrane

Same as section *2.1.14.4.*

2.1.17.5 Washes and stripping of probe from membrane

Same as section *2.1.14.5.*

2.1.18 Complementary DNA preparation for analysis with PCR

The RNA concentration was estimated by absorbance at 260 nm as previously described. cDNA was generated from an RNA template using a First Strand cDNA Synthesis Kit supplied by Boerhinger Mannheim Roche (Indianapolis, IN). For each RNA sample two aqueous solutions containing 1μg of total RNA were made up to 10μl with sterile dH_2O. To one sample 10μl of the 'Reaction Mixture' containing RNAase inhibitor, magnesium chloride, dNTPs, aqueous buffer and 2μl Oligo-$p(dT)_{15}$ primer was added; this was the rt negative control. To the other sample was added the same reaction mixture plus 1μl AMV reverse transcriptase; this was the rt positive

sample. All samples were then incubated at 25°C for 10 minutes and then at 42°C for 60 minutes. For analysis with polymerase chain reaction (rtPCR) 2μl of the reaction mixture was used in each PCR sample. Both rt positive and rt negative samples were first analysed for glyceraldehyde phosphate dehydrogenase (GAPDH) to confirm a lack of DNA contamination of the mRNA and identify equal quantities of input RNA to the rtPCR procedure.

2.1.19 Amplification of DNA sequences by the polymerase chain reaction

Polymerase chain reaction (PCR) was performed by cycling samples containing template DNA mixed with sequence-specific oligonucleotide primers through three temperature incubations in the presence of *Thermus aquaticus* (*Taq*) DNA polymerase; AmpliTaq (Perkin Elmer, CA, USA) was used. These cycles were:

1. Denaturation of double stranded DNA.

2. Annealing of primers to DNA.

3. Extension of target sequences by *Taq* DNA polymerase.

The PCR was carried out in a Biometra TRIO-thermoblock (Biometra, Gottingen, FRG). The optimal cycle number and exact annealing and extension conditions were as described in **Table 2.1**. Primers were synthesised by the Molecular biology Core Facility, Mayo Foundation, on an Applied Biosystems 380B Synthesiser and are listed in Table 2.2. The reaction mixtures were prepared in a laminar flow hood isolated from normal areas of DNA handling. Each reaction sample consisted of: template DNA (1 μg of genomic DNA or 0.1-0.5 μg of plasmid DNA; for semi-quantitative rtPCR the cDNA equivalent of 0.1 μg RNA was used), 8 μl dNTPs (40mM), 5 μl of 10x PCR buffer, 0.2 μg 5' primer, 0.2 μg 3' primer, 0.5 μl *Taq* DNA polymerase (5 units/μl) and dH_2O added to a total volume of 50 μl. The reaction was then heated to 94°C for 10 min and then allowed to proceed through 20 to 30 cycles of denaturation, annealing and extension to produce the required degree of amplification. If the PCR product was required for cloning experiments a final 10 min extension cycle at 72°C was added. The amplified PCR products were evaluated by mixing 12 μl of the reaction mixture with 2 μl of 6x loading buffer stock solution and run on an agarose gel.

2.1.20 Ligation of PCR products

A TA cloning kit (Invitrogen, Carlsbad, CA) was used to ligate PCR products into pCR3.1 vector. This system takes advantage of the nontemplate-dependent activity of *Taq* polymerase which adds a single deoxyadenosine to the 3' termini of the double stranded DNA molecules. The pCR3.1 vector is provided in a linearised form possessing single overhanging deoxythymidine residues at the 3' termini, thus allowing the PCR product to ligate efficiently with the vector. The ligations were performed according to the manufacturer's instructions: 1 μl of PCR reaction mixture was mixed with 1 μl of 10x ligation buffer, 1 μl T4 DNA ligase, 2 μl of linearised vector (60 ng pCR3.1) and 5 μl distilled water and incubated overnight at 14°C. The ligation mixture was then transformed into competent *E.coli* (TOP10F' strain for pCR3.1) and plated onto L-agar containing ampicillin.

2.2 Cell Biology

2.2.1 Eukaryotic cell culture – general procedures

All manipulations involving cell culture were carried out in a sterile environment provided by a laminar flow hood. All tissue culture reagents were filter sterilised by passage through a 0.22 μm filter and stored in sterile autoclaved containers.

The cell lines used in this work are listed in Table 2.2. Adherent cell lines were grown as monolayers in plastic tissue culture flasks or dishes (Nunc, Nalge Nunc, Naperville, IL) in DMEM or RPMI supplemented with 10% v/v heat-inactivated fetal calf serum (Gibco BRL, Life Technologies, Grand Island, NY) and incubated at 37°C in 5 or 10% CO_2. For culture of cells used in the Tet-On system studies Tet system approved fetal bovine serum (Clontech Laboratories Inc, Palo Alto, CA) was used. Cells were grown until just subconfluent (approximately 2 to 4 days) and were subcultured 1:10, using trypsin (0.05% w/v)/5 mM EDTA to detach the cells. Cell counts were performed using an Improved Neubauer haemocytometer and an inverted microscope (Olympus 1X70).

2.2.2 Storage and recovery of cells stored in liquid nitrogen

Cells were trypsinised, pelleted and resuspended at approximately 10^6 cells/ml in filter-sterilised medium containing 10% v/v dimethylsulphoxide (DMSO). 1ml aliquots were transferred to 1.5ml Nunc cryotubes which were then placed within a 1°C Freezing Container (Nalgene) and stored in a -70°C freezer. Using this apparatus, the cells cooled at approximately 1°C per min. Frozen cells were then transferred to liquid nitrogen tanks (-196°C) the following day.

Recovery of cells from liquid nitrogen storage was performed by rapid thawing in a 37°C water bath. Thawed cells were washed in 5 ml of medium, harvested by centrifugation (110*g* for 5 min) and were then transferred to 25 cm^2 flasks containing fresh culture medium.

2.2.3 Gene transfer into eukaryotic cells

2.2.3.1 Growth selection systems

i) Geneticin (G418 sulphate) (Gibco, Life Technologies, Scotland).

Geneticin is an aminoglycoside antibiotic related to Gentamicin and is toxic to both prokaryotic and eukaryotic cells. Introduction of the neomycin phosphotransferase gene into eukaryotic cells can confer resistance to Geneticin added to normal medium. Geneticin was added to DMEM to concentrations previously determined to be optimal for selective growth of each cell line.

ii) Puromycin (Gibco, Life Technologies, Scotland)

Puromycin inhibits protein synthesis in eukaryotic cells by acting as an analogue of aminoacyl-tRNA thus causing premature chain termination. The puromycin-*N*-acetyl-transferase gene from *Streptomyces alboniger* may be expressed in mammalian cells and used as a selectable marker for puromycin resistance.

iii) Hygromycin B (GibcoBRL Life Technologies, Scotland)

Hygromycin B is an aminoglycoside antibiotic produced by *Streptomyces hygroscopicus* and is toxic to both prokaryotic and eukaryotic cells by inhibiting protein synthesis. Cloning and expression of the hygromycin resistance gene

encoding for a kinase that inactivates Hygromycin B through phosphorylation allows selection for resistance to Hygromycin B in both prokaryotic and eukaryotic cells.

2.2.3.2 Effectene transfection

Transfer of plasmid vectors into eukaryotic cells was performed using the Effectene Transfection kit by Qiagen (Valencia, CA) according to the manufacturer's instructions. This method is based on condensing DNA with a non-liposomal lipid. The day before transfection 2-8 x 10^5 cells were seeded per 25 cm^2 flask in 5 ml of fresh medium such that the following day cells were 60-80% confluent. On the day of transfection 1 μg of DNA was made up to 150 μl with the DNA-condensation buffer, Bufer EC. 8 μl of Enhancer were added, the reaction was mixed by vortexing for 1 sec and incubated at room temperature for 5 min. 25 μl of the Effectene Transfection Reagent were then added to the mixture, the mixture vortexed for 10 sec and incubated at room temperature for 5-10 min. 800 μl of cell culture media was then added to the reaction and added in the flask containing 4 ml of fresh medium.

Cells were incubated at 37°C, 10% CO_2 for 48 hrs and were then split in 6 cm plates in selection medium containing the appropriate growth selection antibiotic. 10-14 days later individual single cell clones were lifted off the plates using trypsin-soaked sterile filter paper microsquares, transferred to individual wells of a 24-well plate, and expanded into larger cell culture flasks.

2.2.4 Murine dendritic cell culture

DCs were cultured from bone marrow of C57BL mice according to a protocol modified from that of Inaba et al. (Inaba, Inaba et al. 1992). Briefly, femurs and tibias were extracted from mice freshly-killed by CO_2 inhalation, stripped of muscle and the ends excised. Bone marrow was flushed from the bones, pipetted until single cell suspensions were obtained, filtered through a cell strainer to remove muscle or bone fragments and centrifuged at 1500 rpm for 10 min. Red blood cells were removed by resuspending the pellet in 9 ml of cold dH_2O and adding 1 ml of 10x PBS. 1 volume i.e. 10 ml of RPMI medium was added and the mixture filtered and centrifuged as before. The cells were resuspended in 1 ml of medium containing a mixture of

monoclonal antibodies for the depletion of a range of cells types (see **Table 2.3**) and incubated for 20 min on ice. Cells were washed twice in media, resuspended at a final concentration of 1x 10^7 cells/ml in diluted baby rabbit serum (Cedarlane, Ontario, Canada) and incubated at 37°C for 45 min. Cells were washed twice in media and resuspended at a final concentration of 1x 10^6 cells/ml in culture medium (90% (v/v) RPMI, 10% (v/v) FCS, 10 ng/ml recombinant murine GM-CSF (R&D)) and plated in 75 cm^2 flasks. Cells were cultured for a total of 7 days with 2/3 of the media being replaced on day 3 of the culture. At the end of the culture, non-adherent and loosely adherent cells were harvested, counted and resuspended in appropriate medium for subsequent use.

2.2.5 Murine peritoneal macrophage culture

C57BL/6 mice were euthanised, the abdominal skin was retracted to expose the peritoneal wall and 8 ml of DMEM containing 10% FCS were injected into the peritoneal cavity. Approximately 6 ml of fluid were withdrawn, containing resident peritoneal marophages. The cells were washed and resuspended in medium containing penicillin and streptomycin for further analysis.

2.2.6 Cell staining for β-gal expression

Adherent cells transduced with β-gal were washed twice in PBS and fixed in 1ml fresh formaldehyde solution (4% in PBS) for 10 min at 4°C. After 2 further washes in PBS cells were overlaid with 1ml filter-sterilised (0.45μm) X-gal stain (100 mM sodium phosphate pH 7.3, 1.3 mM $MgCl_2$, 3 mM $K_3Fe(CN)_6$, 3 mM $K_4Fe(CN)_6$, 1 mg/ml X-gal). After incubation overnight at 37°C cells were examined for blue staining by light microscopy.

2.2.7 Immunohistochemistry

Injection sites were surgically excised, fixed in 10% neutral buffered formalin for a minimum of 24 hrs and the Mayo Histology Core facilities performed paraffin embedding, sectioning (5 μm thick sections) and staining with hematoxylin and eosin (H&E).

2.2.8 Analysis of immune cell infiltrates at vaccination site using gelfoam gelatin sponges.

Gelfoam absorbable gelatin sponges (Pharmacia, Upjohn, Kalamazoo, MI) (size 100, precut to 1.5 x 1.5 x 0.5 cm) were hydrated in HEPES buffer and surgically implanted under sterile conditions in the subcutaneous space at the back of C57BL/6 mice (two sponges per mouse). The animals were allowed to recover from the procedure for three days and 5 x 10^5, 100Gy irradiated, tumour cells were directly injected into each sponge. 48 hrs later the sponges were surgically removed, chopped into small pieces using sterile scalpels and incubated in a collagenase-containing buffer (170 μg/ml collagenase, 20 mg/ml bovine serum albumin (BSA), 1% v/v pennicillin/streptomicin in Saline G) at 37°C for 3 hrs in a rotating incubator (225 rpm). (Saline G: 0.11% glucose, 0.13 M NaCl, 5.3 mM KCl, 1 mM Na_2HPO_4 $7H_2O$, 1.1 mM KH_2PO_4, 0.62 mM $MgSO_4$, 0.1 mM $CaCl_2$ $2H_2O$). The dissolved sponges were filtered through a 100 μm cell strainer to remove remaining pieces of sponge and tissue and the cells in suspension were centrifuged at 1500 rpm for 5 min. Red blood cells were removed by incubating the cells in 5 ml of red blood cell lysis buffer (156 mM NH_4Cl, 9.5 mM Na_2CO_3) for 2 min and adding 10 ml of 1x PBS. Cells were counted centrifuged as before and resuspended in FACS wash buffer (see flow cytometry section) for further analysis.

2.3 Assays

2.3.1 Flow Cytometry

Adherent cells were trypsinised and washed once in culture medium. Cells were then washed twice in ice-cold wash buffer (PBS with 0.1% w/v BSA and 0.1% sodium azide) and separated into 1 x 10^6 cells/sample. The cells were then suspended in 100μl wash buffer containing the appropriate conjugated mouse monoclonal antibody (dilution 1:50) and incubated at 4°C for 60 minutes (for list of antibodies used see **Table 2.4**). As a negative control, mouse IgG was used. After washing and spinning down the cells three times with wash buffer, the cell pellet was resuspended in 500μl

of fresh formaldehyde solution (4% in PBS) and stored overnight at 4°C. The cells were then analysed using a Becton Dickinson FACScan and Cellquest software (Becton Dickinson Immunocytometry systems, San Jose, CA).

2.3.2 Murine GM-CSF Enzyme-Linked Immunosorbent Assay (ELISA)

GM-CSF ELISA was performed using OptEIA™ GM-CSF kit from PharMingen (San Diego, CA) according to the manufacturer's instructions. Briefly, microwell plates (Rainin) were coated with 100 µl of capture antibody/well (antibody provided in the kit) diluted to 2 mg/ml in coating buffer (0.1 M Carbonate, pH 9.5) and incubated overnight at 4°C. Plate was washed three times with 300 µl/well wash buffer (PBS with 0.05% Tween-20) and blocked with 200 µl/well assay diluent (PBS with 10% FBS, pH 7.0) by incubating at room temperature for 60 min. The washing procedure was repeated as described above and 100 µl/well of test sample and standards were added in triplicate and incubated for 2 hrs at room temperature. Standards were made up from a stock of 118 ng/ml recombinant mouse GM-CSF: 20 µl of stock was added to 2.36 ml of assay diluent giving an upper standard value of 1 ng/ml. Serial dilutions of 1:2 were then performed down to 15.625 pg/ml. The plate was washed again as described above with a total of 5 washes and 100 µl of working detector (1:1 mixture of detection antibody (diluted 1:250 in assay diluent) and avidin-horseradish peroxidase conjugate (diluted 1:250 in assay diluent)) was added and incubated for 60 min at room temperature. The plate was washed again as described above with a total of 7 washes and 100 µl of substrate solution (tetramethylbenzidine and hydrogen peroxide, provided in the kit) were added and incubated for 30 min at room temperature in the dark. 50 µl of stop solution (2 N H_2SO_4) were added to each well and the absorbance at 450 nm was read within 30 min of stopping the reaction.

2.3.3 IL-12 (p70) ELISA

IL-12 (p70) ELISA was performed using OptEIA™ IL-12 (p70) kit from PharMingen (San Diego, CA) according to the manufacturer's instructions. The procedure used was same as the one used for the murine GM-CSF ELISA described above. Capture

antibody used was an anti-mouse IL-12 antibody diluted 1:250 in coating buffer. The detection antibody used was a biotinylated anti-mouse IL-12 antibody diluted 1:250 in assay diluent. Standards were made up from a stock of 77 ng/ml recombinant mouse IL-12: 20 µl of stock was added to 0.75 ml of assay diluent giving an upper standard value of 2 ng/ml. Serial dilutions of 1:2 were then performed down to 31.3 pg/ml.

2.3.4 IFN-γ ELISA

IFN-γ ELISA was performed using OptEIA™ IFN-γ kit from PharMingen (San Diego, CA) according to the manufacturer's instructions. The procedure used was same as the one used for the GM-CSF ELISA. Capture antibody used was an anti-mouse IFN-γ antibody diluted 1:250 in coating buffer. The detection antibody used was a biotinylated anti-mouse IFN-γ antibody diluted 1:250 in assay diluent. Standards were made up from a stock of 77 ng/ml recombinant mouse IFN-γ: 20 µl of stock was added to 0.75 ml of assay diluent giving an upper standard value of 2 ng/ml. Serial dilutions of 1:2 were then performed down to 31.3 pg/ml.

2.3.5 Luciferase Assay

Detection of luciferase activity was performed using Promega's Luciferase Assay System (Promega, Madison, WI) according to manufacturer's instructions. All solutions were equilibrated to room temperature prior to use. Growth medium was removed from cells and cells were gently washed twice with 1x PBS. 100 µl of 1x cell culture lysis reagent (25 mM Tris-phosphate, pH 7.8, 2 mM DTT, 2 mM 1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid, 10% glycerol, 1% Triton X-100) was added per well of a 6 well plate, the cells were scraped free from the culture dish and transferred with the solution to a microfuge tube. Following a brief (5 sec) centrifugation at 12,000 *g*, 20 µl of the cell-free supernatant were mixed with 100 µl of Luciferase Assay Reagent and the reaction was immediately read in a luminometer (Turner Designs TD-20/20, Sunnyvale, CA) by measuring the light produced for 10 seconds.

2.3.6 Western Blot

2.3.6.1 Preparation of samples

Adherent cells were harvested by trypsinisation and washed twice with 1x PBS. The cells were lysed by adding 200 µl of lysis buffer (425 mM NaCl, 50 mM Tris-HCl pH 8.0, 10 ml Nonidet P-40, 0.4% DOC, 0.1% SDS, 714 µl β-mercaptoethanol, in 1 L of dH_2O, 10 µl/ml protease inhibitors) and incubating on ice for 10 min. The cells were then sonicated for 15 sec, centrifuged at 14,000 rpm at 4°C for 30 min and the supernatant transferred to a clean microfuge tube. Protein concentration was then determined. Protein was then mixed with an equal volume of Laemmli 2x Sample buffer (0.65 M Tris/HCl pH 6.8, 2.1% SDS, 0.38% glycerol, 0.01 % bromophenol blue, 20% β-mercaptoethanol), heated at 95°C for 5 minutes and cooled on ice for 2 minutes.

2.3.6.2 Determination of protein concentration

Protein concentration was determined using a colorimetric assay, DC protein assay (Bio-Rad, Hercules,CA). This assay was conducted as per the manufacturer's instructions in a microplate and is based on the reaction of protein with an alkaline copper tartrate solution followed by the reduction of a Folin reagent; producing a blue colour, measurable at an absorbance of 750 nm using the SPECTRAmax 190 (Molecular Devices) spectrophotometer. Briefly a protein standard was prepared using 1:2 dilutions of BSA; range 1.4 mg/ml-0.088 mg/ml. 'Working reagent A' was produced by adding 20 µl of reagent S (SDS) to 1ml of reagent A (1-5% sodium hydroxide, <1% sodium tartrate, <0.1% copper sulfate). Next 5 µl of standards and samples were pipetted into wells of a microtitre plate and 25 µl of working reagent A added. This was followed by the addition of 200 µl of reagent B (Folin reagent), the plate agitated and incubated for 15 minutes at RT. The absorbance was then read at 750 nm and sample values obtained from the standard curve. All samples were adjusted to 1 mg/ml with lysis buffer.

2.3.6.3 Electrophoresis of samples

10 μg of protein was loaded onto a Tris-HCl precast gel (Bio-rad) and the gel was run in 1x running buffer (1.5 M Tris base, 1.4% w/v glycine, 0.4% w/v SDS, pH 8.0) for 1 hr at 150 V.

2.3.6.4 Transfer of protein onto nitrocellulose membrane

The gel was placed on top of three sheets of Whatmann 3mm paper pre-soaked in 1x transfer buffer (3 M Tris Base, 0.3% w/v glycine, 0.0375% w/v SDS) on the positive electrode of a semi-dry blotting aparatus (Trans-blot SD, Bio-Rad). A sheet of Hybond-C + nitrocellulose membrane (Amersham pharmacia biotech, Buckinghamshire, UK), pre-soaked in dH_2O followed by 1x transfer buffer, was placed in direct contact with the gel, followed by 3 more sheets of 3MM paper pre-soaked in 1x transfer buffer and the negative electrode plate. Transfer was allowed to take place for 30-40 min at a constant current of 20 V.

2.3.6.5 Blocking, antibody and chemiluminescence

The membrane blot was blocked in 40 ml of 10% w/v milk in 1x TBS-Tween (10x TBS: 0.1M Tris-HCl pH 7.4, 1.5 M NaCl) (diluted TBS to 1x with dH_2O and added 0.05% v/v Tween-20/500 ml) by incubating at room temperature for 2 hrs with gentle shaking. The blot was then washed twice with 1x TBS-Tween, diluted primary antibody added to it and incubated with gentle shaking for 3 hrs at room temperature or overnight at 4°C. Primary antibody was prepared in 1x TBS-T, 1% BSA, 0.1% Na Azide. For antibodies used see **Table 2.5**. The blot was washed with three times with 1x TBS-Tween by gentle shaking at room temperature, diluted secondary antibody added to it and incubated with gentle shaking for 2 hrs at room temperature or overnight at 4°C. Secondary antibody was prepared in 1x TBS-Tween. Following extensive washing with 1x TBS-Tween, bands were revealed using the enhanced chemiluminescence (ECL) system (SuperSignal West Pico Chemiluminescent substrate, Pierce, Rockford, IL). This system requires the mixing of equal volumes of a stable peroxide solution and luminol/enhancer solution prior to covering the blot and incubating for 5 minutes. The blot was then removed, placed in a plastic

protector and placed against Kodak film for an exposure of 30 seconds to 5 minutes prior to developing.

2.3.7 Syncytia Formation Induced by FMG Expression

2-5x 10^5 cells were plated per well of a 6 well plate. The following day, the cells were transfected with 1 μg of plasmid expressing the VSV-G or GALV genes using the Effectine transfection protocol described previously. In the case of VSV-G, 24 hrs later the cells were incubated in 2 ml medium of pH 5.5-5.7 for 2 min, washed three times in PBS and returned to normal culture medium. 24-48 hrs following the pH drop in the case of VSV-G or transfection in the case of GALV extensive syncytia formation was observed by light microscopy.

Percent of syncytia formation was determined by dividing the number of cells involved in syncytia by the total number cells per visual field. For each sample, at least 6 different visual fields were examined and the average % of fusion was determined. A + and - grading system was created to indicate the extent of fusion:

-	0 % fusion
+	≤ 20% fusion
++	≤ 40% fusion
+++	≤ 60% fusion
++++	≤ 80% fusion
+++++	≤ 100% fusion

2.4 Animal Studies

All animal studies presented in this thesis were approved by the Institutional Animal Care and Use Committee at the Mayo Foundation.

2.4.1 Mice

6-8 week old female C57BL/6 mice (Jackson Laboratory, Bar Harbor, Maine) and 4-5 week old NCr (*nu/nu*) nude female mice (Taconic, Germantown, NY) were used in

this thesis and were housed in the biological resources units at Mayo Foundation (Rochester, MN). Mice were matched for age and sex for individual experiments. Standard food and water were provided *ad libitum.* Between 8 and 10 mice were used per group in each experiment. For challenge with live tumour cells, all groups of mice in any one individual experiment were rechallenged on the same occasion using the same preparation of cells. A naive group of mice was also injected with these cells at the same time.

2.4.2 Vaccination of mice using irradiated tumour cells

On day 1 tumour cells were prepared, γ-irradiated to 100Gy and resuspended in ice-cold PBS at the desired concentration. Mice were injected subcutaneously at the flank with 100 µl of the irradiated cell suspension. This vaccine was repeated on day 8 and 15, and on day 22 mice were challenged on the opposite flank with live parental cells as described in Results for individual experiments. The diameter of tumours, in 2 dimensions, was measured twice weekly using callipers and mice were killed if tumour size progressed to 1.0 x 1.0cm.

2.4.3 Therapy of animals carrying 3 day pre-established tumours

On day 1 tumour cells were prepared in ice-cold PBS and 100 µl of the cell suspension was injected subcutaneously at the flank of mice. Three days later mice were vaccinated subcutaneously with 100 µl of irradiated cells, prepared as previously described. The vaccination was repeated on days 5 and 6 for a total of 3 vaccines. Tumour growth was monitored as above and mice were killed if tumour size progressed to 1.0 x 1.0cm.

2.4.4 Statistical analysis

Data from animal experiments were analysed by plotting Kaplan-Meier curves using the 'occurring event' as the time at which tumour size was equal to or exceeded 1cm in diameter. A tumour was considered present when a palpable mass >0.2cm was noted. Different groups of mice were compared using the logrank test (Altman 1991).

Table 2.1: List of primers and conditions used for PCR amplification of DNA sequences

Amplified Sequence	Forward Primer Reverse Primer	Annealing Temperature	Cycles
Tyr	CAATGGGTCAACACCCATGTTTA GATCCCTGTACTTGGGACATTGTTC	54	30
TRP-1	GTGAGAAGGGATTAGTGAGAGCTGG GAGAAGACAGGGGTGCTCAGATG	58	30
TRP-2	CCATTGATTTCTCTCACCAAGGG TCTCTTGCTGCTGAGACCTGTCTC	55	30
gp100	ACATTTCATCACCAGGGTGCC CAGGAACAAGTTGGGTGCT	54	30
Mage A1	CTATCCAGCCTACTGTTACC CACTCAGTACAGCTATGAGG	51	30
Mage A2	GAGTGCTCAGGCCCAACAGG GGGTACAGCAATGGAATCAGTGAC	57	30
Mage A4	CTATACTGTCCATTGTTACAGGAA CGTGAAAATAGCACCAAGAACAA	51	30
Mage A7	CTCTTCTCCCTGTGTGATGA CTCTCAGTGAGGATACTTTTCTG	51	30
IFN-γ	TCTGAGACAATGGCCGCTACACACTGC GTCGACTCTTATTGGGACAATCTCTT	56	30
TNF-α	TTCTATGGCCCAGACCCTCACA GATGAACACCCATCCCCTTCAC	54	30
Neo	AGACAATCGGCTGCTCTGAT CGGCCATTTTCCACCATGAT	51	30
GAPDH	TGAAGGTCGGTGTGAACGGATTTGCG CATGTAGGCCATGAGGTCCACCAC	58	30
GALV (*Bam*HI)	GGATCCGGATCCGGTGGCCCTCCTATAGTGAG GGATCCGGATCCTAAGCCTGGTACCGTAACAA	58	35

Table 2.2: List of cell lines used in this thesis

Cell Line		Reference
K1735	murine melanoma	[Kripke, 1979]
K1735-IL-12	murine melanoma	R. Vile
K1735-GM-CSF	murine melanoma	R. Vile
K1735-IFN-γ	murine melanoma	R. Vile
K1735-hsp70	murine melanoma	R. Vile
K1735-neo	murine melanoma	R. Vile
B16-F1	murine melanoma	[Fidler, 1975]
A375M	human melanoma	[Giard, 1973]
293-On	human kidney embrional	Clontech
Hela	human cervical carcinoma	R. Vile
Hela-On	human cervical carcinoma	Clontech
Mel624	human melanoma	R. Vile
Mel888	human melanoma	R. Vile

Table 2.3: List of monoclonal antibodies used for immune cell depletion

Monoclonal Antibody	Antigen source	
KH74	I-Ab	Pharmingen
145-2C11	CD3e	Pharmingen
M3/84	Mac3	Pharmingen
53-6.7	CD8a	Pharmingen
RB6-8C5	Gr-1	Pharmingen
RA3-6B2	CD45R	Pharmingen

Table 2.4: List of monoclonal antibodies used for cell surface staining

Monoclonal Antibody	Type	Antigen source	Conjugation	
AF6-120.1	mouse	I-Ab	FITC	Pharmingen
AF6-120.2	mouse	I-Ab	PE	Pharmingen
11.5.2	mouse	I-Ak	PE	Pharmingen
KH95	mouse	H-2Db	FITC	Pharmingen
15-5-5	mouse	H-2Dk	FITC	Pharmingen
AF6-88.5	mouse	H-2Kb	FITC	Pharmingen
AF6-88.6	mouse	H-2Dk	PE	Pharmingen
36-7-5	mouse	H-2Kk	PE	Pharmingen
145-2C11	hamster	CD3	FITC	Pharmingen
RM4-5	rat	CD4	PerCP	Pharmingen
55-5.8	rat	CD8a	PE	Pharmingen
HL3	hamster	CD11c	FITC	Pharmingen
HL4	hamster	CD11c	PE	Pharmingen
M5E2	mouse	CD14	PE	Pharmingen
30-F11	rat	CD45	PerCP	Pharmingen
16-10A1	rat	CD80	FITC	Pharmingen
GL1	rat	CD86	FITC	Pharmingen
3 E2	hamster	ICAM-1	FITC	Pharmingen
RB6-8C5	rat	Ly6-G	PE	Pharmingen
M3/84	rat	Mac3	PE	Pharmingen
PK136	mouse	NK1.1	PE	Pharmingen
H-2.65	mouse	H-2Ld	-	Cedarlane

Table 2.5: List of antibodies used in Western Blotting

Anibody	Type	Antigen source	
primary			
SPA-810	mouse	hsp-70	StressGen
sc-8432	mouse	actin	Santa Cruz Biotech.
secondary			
NA931V	sheep	α-mouse Ig	AmerSham Pharmasia

Chapter 3

MODULATION OF THE IMMUNE RESPONSE AGAINST B16 MELANOMA BY CYTOKINE- OR HSP70- EXPRESSING ALLOGENEIC K1735 TUMOUR CELL VACCINES

The use of whole tumor cells in cancer vaccine preparation has been extensively explored in recent years. The primary advantage of such vaccines is their potential to be a single and direct source of the entire spectrum of tumor associated antigens (Dranoff, Jaffee et al. 1993; Simons, Jaffee et al. 1997; Soiffer, Lynch et al. 1998; Schadendorf, Paschen et al. 2000; Jaffee, Hruban et al. 2001). However, the technical difficulties and high cost of production currently associated with the production of individualized cancer vaccines have hindered the widespread clinical use of autologous cancer vaccines. On the other hand, increasing interest has been developed in using well established, well characterized, easily modifiable and standardized cell lines in cancer vaccines (Chan and Morton 1998; Fabre 2001). Such generic vaccines can be optimally designed to share expression of significant amounts of immunologically important tumor antigens with the target tumour (Morton, Hoon et al. 1993; Habal, Gupta et al. 2001). The use of allogeneic vaccines is supported by the finding that antigens from allogeneic cells can be processed and presented by host APC to activate host T cells (Huang, Golumbek et al. 1994; Mitchell, Nair et al. 1998; Ochsenbein, Klenerman et al. 1999). Furthermore, the allogeneic nature of such vaccines may in itself offer an immunological advantage in the form of an adjuvant effect, where the allogeneic component of the cells (predominantly the allogeneic MHC molecules) may stimulate a non-specific immune response, which can enhance the immune response to the tumor antigens in the vaccine (Fabre 2001).

To investigate potential mechanisms of enhancing the efficacy of allogeneic vaccines in melanoma, K1735 melanoma cells were used as an allogeneic vaccine against the development of B16 melanoma. K1735 is an MHC class I low expressing melanoma derived from H-2^k C3H/He mice (Kripke 1979) and B16 is a MHC class I negative melanoma which grows in H-2^b C57BL/6 mice (Fidler 1975). This model was chosen because we, and others, have previously shown that K1735 cells by themselves are only very poor immunogens against B16 tumor growth in C57BL/6 mice (**Figure 3.1** and (Kayaga, Souberbielle et al. 1999; Melcher, Todryk et al. 1999; Todryk, Birchall

et al. 2001), allowing significant scope for improvement by manipulation of the cellular vaccine. The autologous B16 cells are only weakly immunogenic against B16 tumour growth (Figure 3.1, P= 0.0018 compared to PBS group).

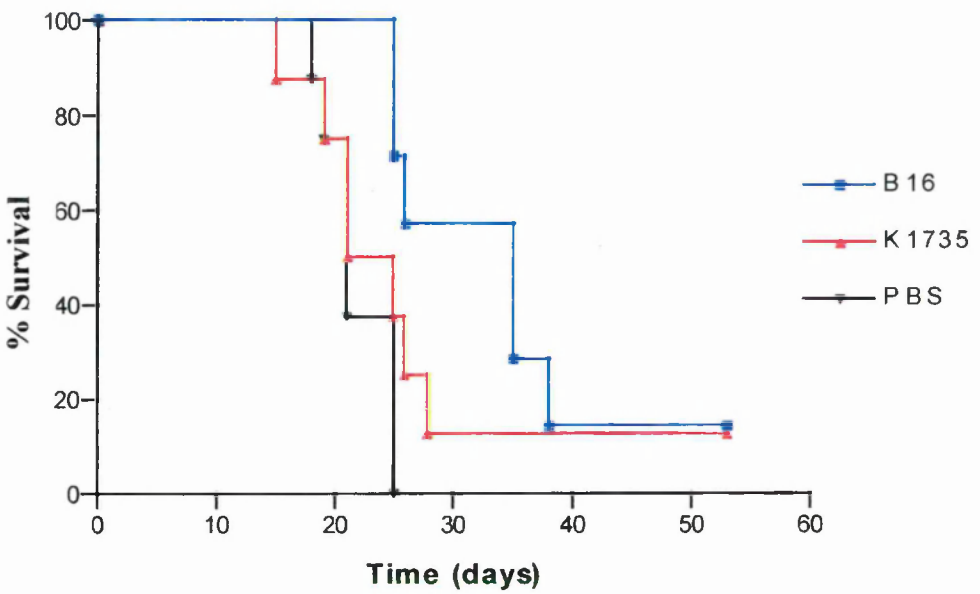


Figure 3.1: Irradiated allogeneic K1735 cells as a vaccine against the development of B16 melanoma.
Groups of 8 C57BL/6 mice received 3 sequential subcutanous injections of $5x10^5$ irradiated (100Gy) K1735 cells or PBS at days 1, 8 and 15. At day 22 (day 0 on the graph) mice received an injection of $2x10^5$ live B16 cells on the opposite flank and tumour size was followed. Animals were sacrificed once tumour size exceeded 1cm in diameter. Percentage of animals surviving the tumour challenge are shown.

Genetic modification with immune stimulating cytokines as well as HSPs has been shown to augment vaccine efficacy in the B16 and other models (Dranoff, Jaffee et al. 1993; Chong 1996; Rakhmilevich, Turner et al. 1996; Chong, Todryk et al. 1998; Nawrocki and Mackiewicz 1999). Therefore, as a first step K1735 cells were modified to express a selection of immunostimulatory cytokines (IL-12, GM-CSF, IFN-γ), or hsp 70, and the effect of these proteins on the efficacy of the allogeneic vaccine to generate immune protection against the syngeneic B16 tumour challenge was studied (For extensive discussion on IL-12, GM-CSF, IFN-γ and hsp70 please see Introduction and Discussion).

3.1 Genetic modification of K1735 cells to express IL-12 does not enhance the efficacy of the allogeneic vaccine against a B16 challenge

IL-12 is a pro-inflammatory cytokine that has been shown to stimulate DC, as well as $CD4^+$ and $CD8^+$ T cells and to induce cell mediated immunity. K1735 cells expressing IL-12 had been previously established in the laboratory using retroviral mediated gene transfer (Chong 1996). The IL-12 secretion levels by the K1735-IL-12 cells was determined by ELISA to be approximately 8.3 ng/$4x10^5$ cells/48hrs, comparable to levels described elsewhere in the literature. The K1735-IL-12 cells were used in prophylactic vaccination experiments where groups of 8 C57BL/6 mice were subcutaneously vaccinated with $5x10^5$ cells, irradiated with 100Gy, on days 1, 8 and 15. 7 days following the last vaccination the mice were challenged on the opposite flank with $2x10^5$ live B16 cells, previously determined as a lethal dose (**Figure 3.2**). All of the mice vaccinated with K1735 unmodified cells succumbed to tumour growth, as did the mice vaccinated with the control cell line K1735-pBabeNeo and the naïve mice. In addition, three vaccinations of K1735-IL-12 cells were unable to protect any animals from the lethal challenge in this experimental model (difference between any of the survival curves did not reach statistical significance).

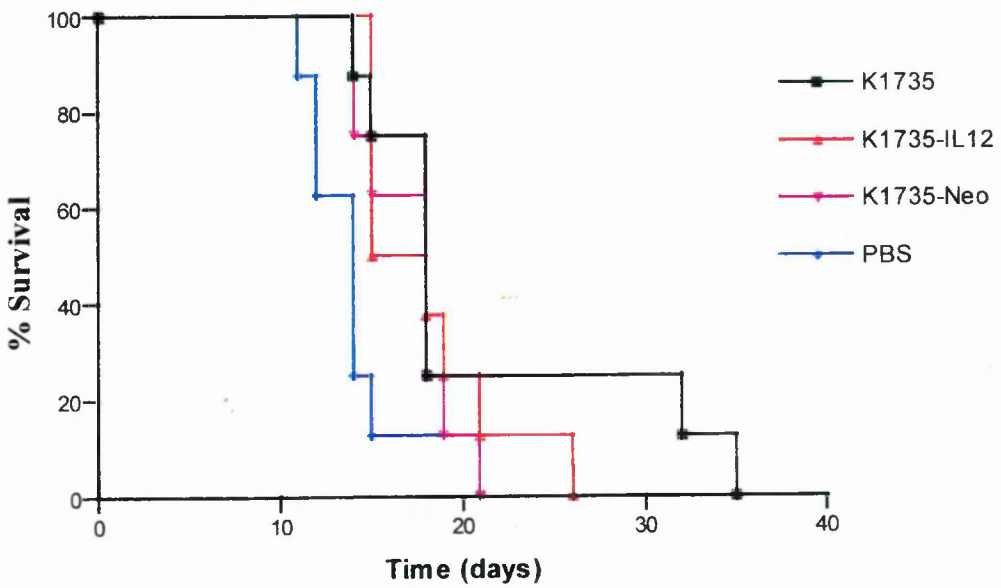


Figure 3.2: Irradiated allogeneic K1735-IL12 cells as a vaccine against the development of B16 melanoma.
Groups of 8 C57BL/6 mice received 3 sequential subcutanous injections of $5x10^5$ irradiated (100Gy) K1735, K1735-IL12, 1735-Neo or PBS at days 1, 8 and 15. At day 22 (day 0 on the graph) mice received an injection of $2x10^5$ live B16 cells at the opposite flank and mice survival was followed.

3.2 Genetic modification of K1735 cells to express GM-CSF does not enhance the efficacy of the allogeneic vaccine against a B16 challenge

A similar set of experiments was conducted to study the effect of GM-CSF on the immunogenicity of the K1735 vaccine. GM-CSF can attract host APC to the tumor site and lead to upregulation of MHC and co-stimulatory molecules on APC, and has been previously shown by our laboratory, and others, to efficiently stimulate protective antitumour immunity (Dranoff, Jaffee et al. 1993; Castleden, Chong et al. 1997; Soiffer, Lynch et al. 1998; Kayaga, Souberbielle et al. 1999; Todryk, Birchall et al. 2001). A K1735 cell line expressing GM-CSF was previously developed in the laboratory (Chong, Todryk et al. 1998). The level of GM-CSF secretion by the K1735-GM-CSF were determined by ELISA to be approximately 1.4ng/4x10^5 cells/48hrs. In a prophylactic vaccination experiment identical to the one described above for **Figure 3.2**, repeated vaccination of C57BL/6 mice with irradiated K1735-GM-CSF had no therapeutic effect against a lethal B16 challenge (**Figure 3.3**). These results are in agreement with other studies of GM-CSF modified allogeneic vaccines (Todryk, Birchall et al. 2001).

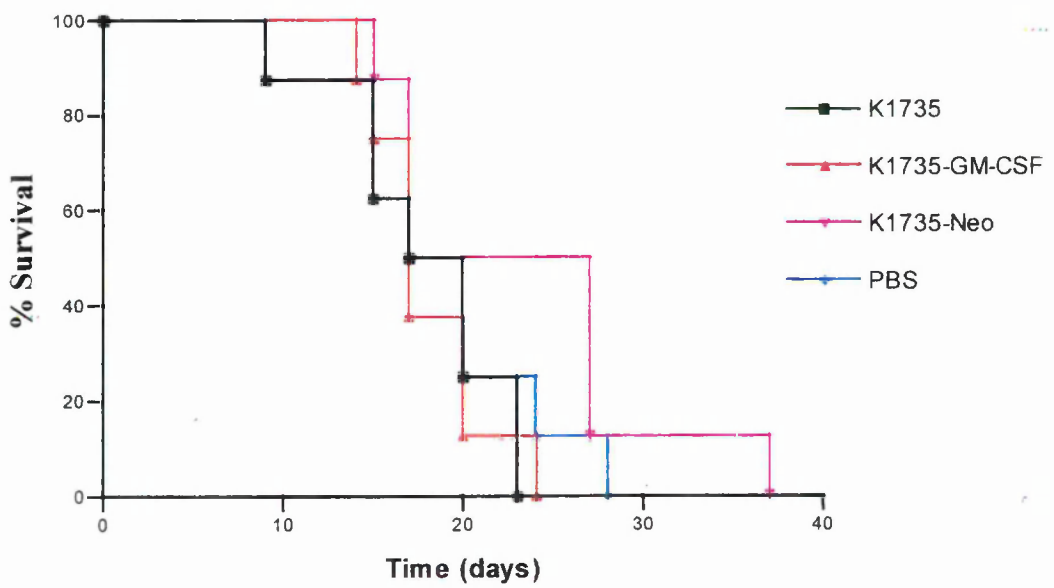


Figure 3.3: Irradiated allogeneic K1735-GM-CSG cells as a vaccine against the development of B16 melanoma.
Groups of 8 C57BL/6 mice received 3 sequential subcutanous injections of 5x10^5 irradiated (100Gy) K1735, K1735-GM-CSF, 1735-Neo or PBS at days 1, 8 and 15. At day 22 (day 0 on the graph) mice received an injection of 2x10^5 live B16 cells at the opposite flank and survival of mice was followed.

3.3 Genetic modification of K1735 cells to express IFN-γ does not enhance the efficacy of the allogeneic vaccine against a B16 challenge

IFN-γ is a cytokine that is associated with Th1 cell mediated immunity, and has been shown to upregulate expression of MHC and co-stimulatory molecules in a range of cell types. IFN-γ-secreting K1735 cells previously developed in the laboratory (Chong 1996) were used as an allogeneic vaccine in vaccine/challenge experiments identical to the ones described above. Levels of cytokine secretion were determined by ELISA to be approximately 3.7 ng/4x10^5 cells/24hrs. **Figure 3.4** shows that IFN-γ release at the vaccine site had no significant effect on the immunogenicity of the K1735 vaccine and conferred no significant survival advantage to the animals over the parental, unmodified, K1735 vaccinated group or the untreated group.

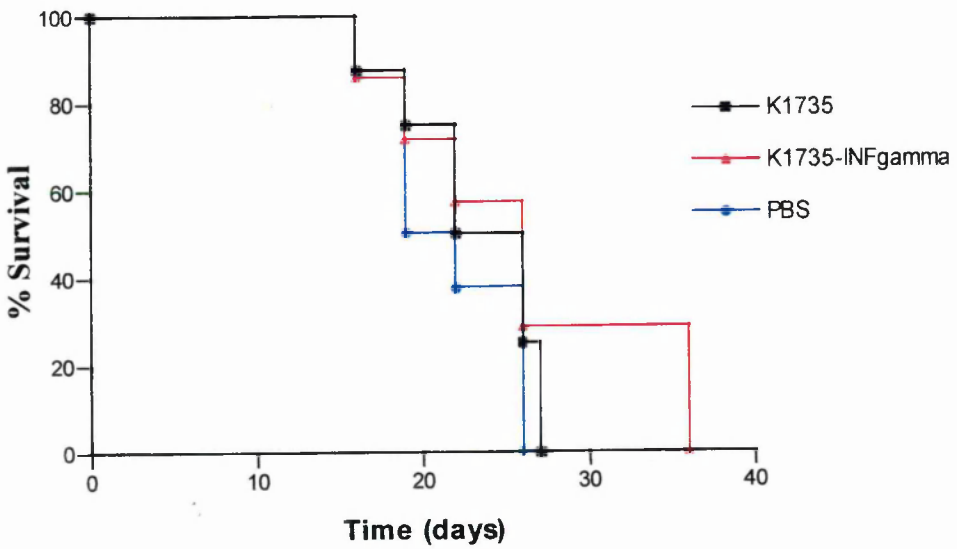


Figure 3.4: Irradiated allogeneic K1735-IFNγ cells as a vaccine against the development of B16 melanoma.
Groups of 8 C57BL/6 mice received 3 sequential subcutanous injections of 5x10^5 irradiated (100Gy) K1735, K1735-IFN-γ, or PBS at days 1, 8 and 15. At day 22 (day 0 on the graph) mice received an injection of 2x10^5 live B16 cells at the opposite flank and animal survival was followed.

The beneficial effect of cytokine secretion by autologous tumour cell vaccines has been well documented in the literature (Cavallo, Di Pierro et al. 1993; Allione, Consalvo et al. 1994; Nawrocki and Mackiewicz 1999; Schadendorf, Paschen et al. 2000). In the melanoma model, expression of GM-CSF, amongst other cytokines, by B16 tumor cells has proven particularly effective in raising a specific and long lasting immune response against B16 tumour growth (Dranoff, Jaffee et al. 1993; Castleden, Chong et al. 1997; Chong, Todryk et al. 1998; Todryk, Birchall et al. 2001). Our findings in the allogeneic vaccination model for melanoma suggest that cytokine modification does not have the same effect on the therapeutic potential of an allogeneic vaccine as that seen with autologous vaccines. As all of our vaccines had to be irradiated due to safety reasons prior to injection in the mice, one possible reason for the apparent lack in generating protective immunity in our model could be the loss of cytokine expression after lethal irradiation of the cells. To test for this possibility, levels of cytokine secretion were determined post irradiation for the K1735 cytokine secreting cell lines studied above. $4x10^5$ cells per well were plated in 6 well plates and the cells were exposed to a total of 100Gy irradiation. Cytokine secretion was followed for a period of 96-144 hrs by harvesting the supernatants from each well and replacing with fresh media every 24 hrs. ELISA for each cytokine was used to determine cytokine levels (**Figure 3.5**). Irradiation did not affect the levels of secretion of IL-12 or GM-CSF by the K1735-IL12 and K1735-GM-CSF cells respectively. Although levels of IFF-γ secretion by irradiated K1735-IFN-γ cells were lower compared to levels obtained from un-irradiated K1735-IFN-γ cells, they remained stable up to 96hrs post irradiation.

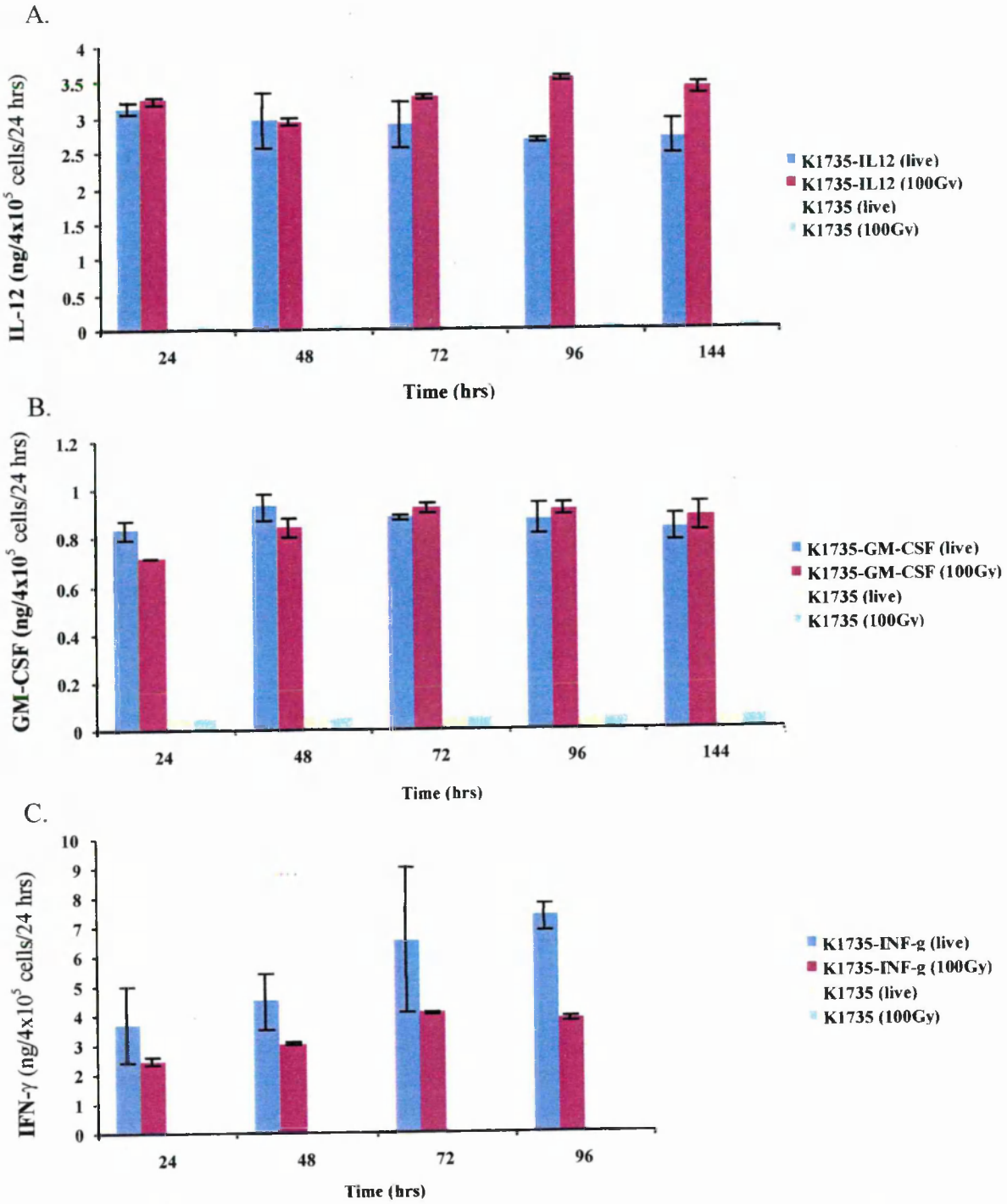


Figure 3.5: Levels of cytokine secretion by genetically modified K1735 cells following irradiation.

A: $4x10^5$ cells/well of K1735-IL12 or K1735 cells were plated in 6 well plates and exposed to 0 (live) or 100Gy irradiation. Supernatants from each well were harvested every 24 hrs (replacing with fresh media) for a total of 144 hrs and concentration of cytokine determined by ELISA. All samples prepared in triplicates. **B:** Same as A for K1735-GM-CSF. **C:** Same as A for K1735-IFNγ. IFN-γ secretion followed up to 96 hrs (*P<0.03).

3.4 Generation of allogeneic tumour cell line overexpressing the inducible form of hsp70 and up-regulation of hsp70 expression in parental cell by heat shock

Previous studies in the laboratory have shown that hsp70 over-expression by B16 tumour cells is associated with increased tumor cell infiltrates, a TH1 profile of intratumoural cytokine production, enhanced antigen uptake by DC and enhanced immunogenicity of a B16 tumour cell vaccine (Melcher, Todryk et al. 1998; Todryk, Melcher et al. 1999). Furthermore, hsp70 has been shown to efficiently chaperone tumour antigens for effective anti-tumour immune responses (Blachere, Li et al. 1997; Castellino, Boucher et al. 2000). Based on these findings, we decided to investigate the effect of hsp70 on the immunogenicity of K1735 cells in our allogeneic prophylactic vaccination model.

K1735 cells were retrovirally transduced with pBabe-hsp70 and a stable pooled population was generated by Puromycin selection (K1735-rhsp70). Hsp70 expression of the Puro resistant pooled population was verified by Western Blot analysis using an anti-hsp70 antibody specific for the inducible form of hsp70. **Figure 3.6A** confirms the expression of high levels of hsp70 by the K1735-rhsp70 cell line (lanes3 & 4), while no detectable hsp70 protein was expressed by the parental K1735 cells (lane 1) or the control K1735-pBabePuro (lane 2).

Hsp expression is upregulated in cells in response to heat stress. Therefore, in addition to generating a stable hsp70-expressing cell line, hsp expression was induced in K1735 cells by heat shock, as described in the Materials and Methods. Briefly, $2x10^5$ cells were plated per well in a 6 well plate, and were exposed to either 42^0C for 20min or 45^0C for 45min, corresponding to sub-lethal or lethal heat shock, respectively. At 4, 6, 12, 24 and 48 hrs following heat shock cells were harvested, protein isolated and hsp expression determined by western blot analysis using an antibody specific for the inducible form of hsp70 (**Figure 3.6B**). K1735 cells heat shocked under sub-lethal conditions (42^0C for 20min) upregulated expression of Hsp70 within 4 hrs of heat shocking and maintained high hsp70 expression levels until 24hrs post heat-shock. By 48 hrs hsp70 levels had reduced significantly. In

contrast, under lethal heat-shock conditions (45^{0}C for 45min) K1735 cells upregulated hsp70 expression more slowly. No hsp70 could be detected at 4 and 6 hrs following heat-shock, while significantly elevated levels of the protein could be detected by 12hrs and were maintained at least until 48hrs. K1735 cells that had not been heat-shocked expressed no detectable hsp70. This data suggested that the optimal timepoints for harvesting K1735 cells following sub-lethal or lethal heat-shock which would result in highly upregulated levels of hsp70 expression were 4-6hrs and 16-24hrs respectively.

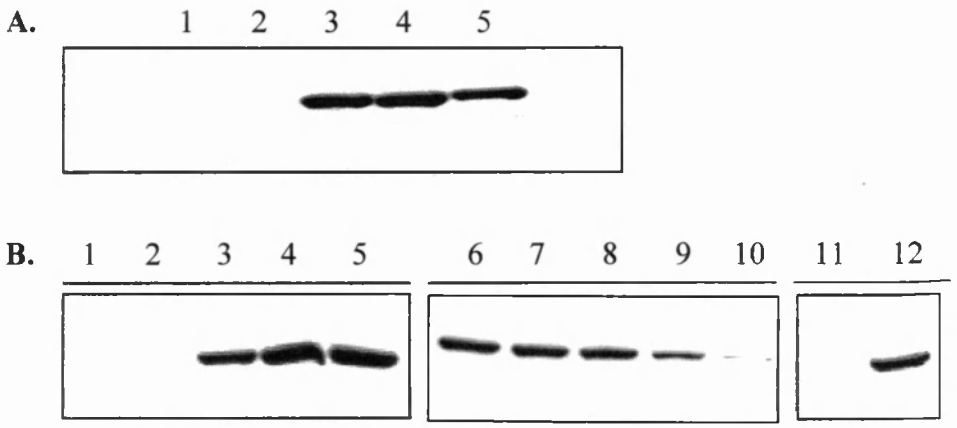


Figure 3.6: Western blot analysis of hsp70 expression in allogeneic K1735 modified cells.
A: Puro resistant K1735 cells infected with pBabe-hsp70 retroviral vector were expanded and protein extracted for Western blot analysis with an anti-hsp70 (inducible) antibody. Lane1– K1735; Lane 2- K1735-pBabePuro; Lane 3- K1735-rhsp70 (passage 8); Lane 4- K1735-rhsp70 (passage 1); Lane 5- positive control for hsp70. **B:** K1735 cells were exposed to lethal (Lanes 1-5) or sublethal (Lane 6-10) heat shock and protein was extracted at 4 (lanes 1 & 6), 6 (lanes 2 & 7), 12 (lanes 3 & 8), 24 (lanes 4 & 9) and 48 hrs (lanes 5 & 10) following heat shock for western blot analysis as in A. Lane 11- K1735; Lane 12- K1735-rhsp70.

3.5 Genetic modification of K1735 cells to express hsp70 does not enhance the efficacy of the allogeneic vaccine against a B16 challenge

K1735 overexpressing hsp70 or sub-lethally or lethally heat-shocked K1735 cells were used as a vaccine against B16 in a prophylactic vaccination protocol identical to the ones previously described. Hsp70 over-expression, either by genetic modification (**Figure 3.7**) or induced by heat-shock (**Figure 3.8**) had no effect on the immunogenicity of an allogeneic K1735 vaccine in our model and had no protective effect against B16 development.

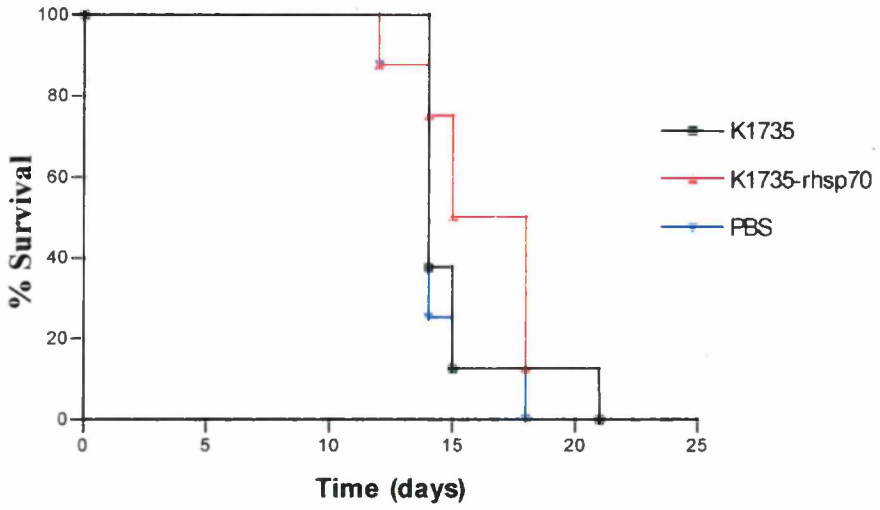


Figure 3.7: Irradiated allogeneic K1735-hsp70 cells as a vaccine against the development of B16 melanoma.
Groups of 8 C57BL/6 mice received 3 sequential subcutanous injections of $5x10^5$ irradiated (100Gy) K1735, K1735-hsp70 or PBS at days 1, 8 and 15. At day 22 (day 0 on the graph) mice received an injection of $2x10^5$ live B16 cells at the opposite flank and animal survival was followed.

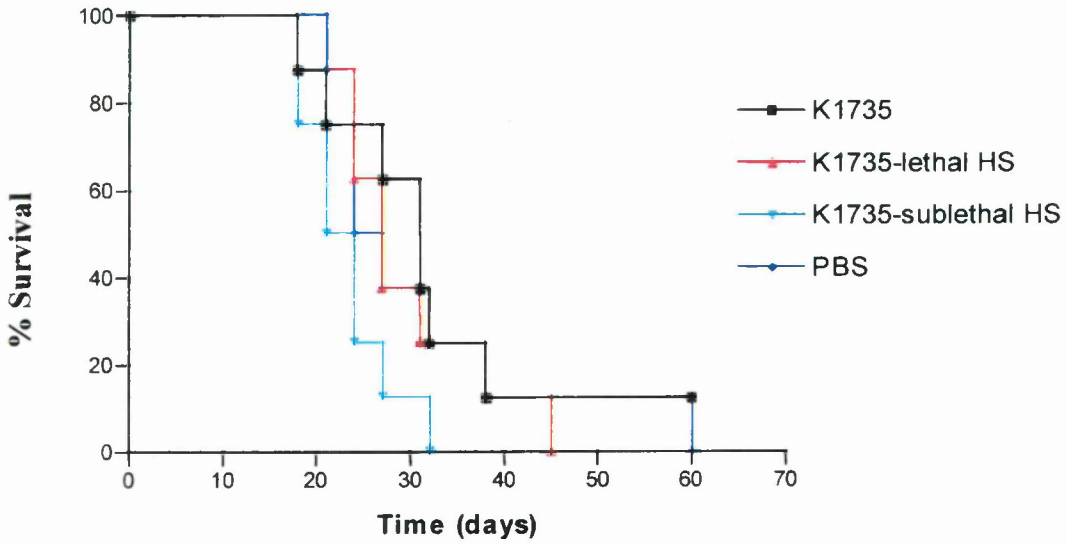


Figure 3.8: Sub-lethally or lethally irradiated allogeneic K1735 cells as a vaccine against the development of B16 melanoma.
Groups of 8 C57BL/6 mice received 3 sequential subcutanous injections of $5x10^5$ irradiated (100Gy) K1735, K1735-hsp70 or PBS at days 1, 8 and 15. At day 22 (day 0 on the graph) mice received an injection of $2x10^5$ live B16 cells at the opposite flank and animal survival was followed.

3.6 Summary

IL-12, GM-CSF or IFN-γ expression by allogeneic K1735 melanoma cells did not enhance the immunogenicity of the allogeneic vaccine in a prophylactic vaccination model against the autologous B16 melanoma tumour. Similarly, hsp70 over-expression by the allogeneic vaccine was ineffective as a treatment against B16 development in the same experimental model.

Chapter 4

Mechanism of Immunogenicity of the K1735 allogeneic Melanoma Cell Line

The data presented in Chapter 3 suggested that the K1735 cells are, at best, only very poorly immunogenic as vaccines against B16 tumours, either when used as parental/unmodified cells or following genetic modification with potentially immunostimulatory genes. To understand the reasons behind this apparent lack of immunogenicity of the allogeneic K1735 cells in our model system, the immunological profile of K1735 cells was analysed. Expression of important immunological markers by the K1735 and the B16 cells was studied. These markers included MHC class I and MHC class II molecules, co-stimulatory molecules, adhesion molecules, and TAA. In addition, the host immune response against the K1735 vaccine was studied by analysing the cytokine profile, as well as the profile of infiltrating immune cells, at the site of injection of the K1735 vaccine.

4.1 Comparative analysis of immunological markers expressed by K1735 and B16 cells

Molecules involved in direct antigen presentation (MHC Class I and Class II), co-stimulatory molecules and adhesion molecules play an important role in the immunotherapeutic potential of a cell-based vaccine. The two melanoma cell lines used in our studies, K1735 and B16, were analysed by FACS for constitutive cell surface expression of MHC Class I and Class II (**Figure 4.1**). K1735 expressed high levels of the H-2K^k and lower levels of the H-2D^k MHC Class I molecules (**Figure 4.1Aiv** and **iii** respectively). B16 expressed neither of the two MHC I molecules (H-2K^b and H-2D^b) tested (**Figure 4.1Aii** and **i** respectively). Neither of the two murine melanoma cell lines showed any expression of MHC Class II on the cell surface (**Figure 4.1B**). Constitutive surface expression of the co-stimulatory molecules B7.1 and B7.2 was also analysed. K1735 cells expressed very low levels of B7.1, but no B7.2 on the cell surface (**Figure 4.1Ciii** and **iv** respectively), while B16 cells expressed neither (**Figure 4.1Ci** and **ii**). Finally, constitutive expression of the

intracellular adhesion molecule (ICAM-1) was analysed and neither of the two cell lines expressed any detectable levels on their cell suface (**Figure 4.1Di** and **ii**).

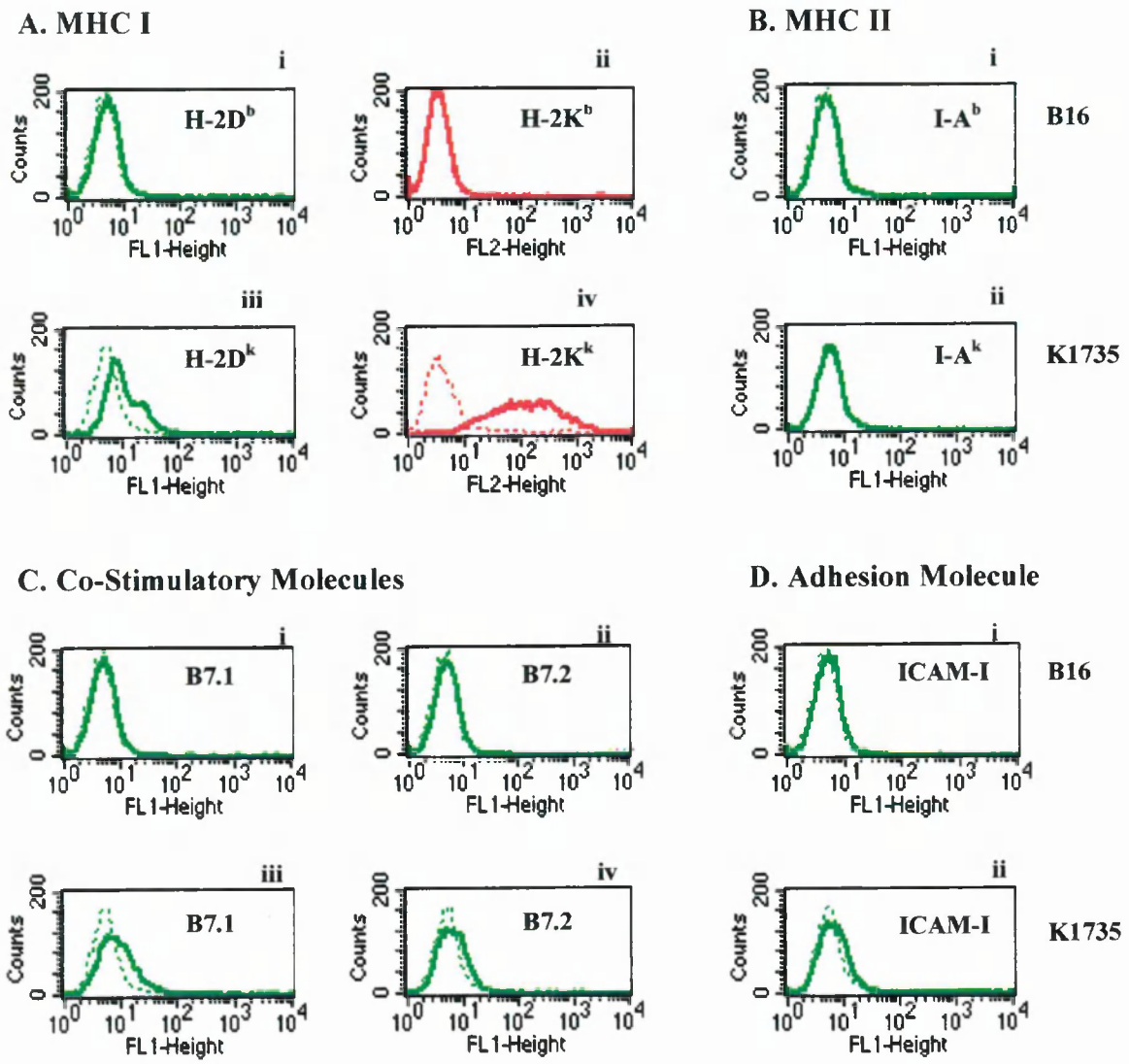


Figure 4.1: Constitutive cell surface expression of immunological markers on K1735 and B16 cells.

FACS analysis of surface expression of A: MHC Class I, B: MHC Class II, C: Co-stimulatory molecules and D: ICAM-I on B16 (Ai, Aii, Bi, Ci, Cii and Di) and K1735 (Aiii, Aiv, Bii, Ciii, Civ and Dii) tumour cells. Dashed lines: control antibody staining; solid lines: surface markers. Green lines: FITC conjugated antibody; Red lines: PE-conjugated antibody

4.2 Comparative analysis of melanoma associated antigen expression by K1735 and B16 cells

Expression of melanoma associated antigens against which a cell mediated immune response can be raised is a key feature of a successful melanoma vaccine. K1735 and B16 cells were screened by rtPCR for expression of a panel of known melanoma associated antigens, which included Tyrosinase, TRP-1, TRP-2, gp100, MAGE-1, MAGE-2, MAGE-4 and MAGE-7. **Table 4.1** shows a qualitative measure of the presence or absence of TAA expression in each cell line. B16 cells constitutively expressed Tyr, TRP-1, TRP-2, gp100, MAGE-2 and MAGE-4. No MAGE-1 or MAGE-7 expression could be detected by rtPCR. In contrast, K1735 cells expressed fewer of the antigens tested. No Tyr, TRP-1, MAGE-1 or MAGE-7 expression could be detected. TRP-2, gp100, MAGE-2 and MAGE-4 were expressed. These data suggest that a relative paucity of shared antigens between the cell lines may contribute to the poor vaccine efficacy of the K1735 cell vaccine. The results of MHC, ICAM-1 and TAA expression by K1735 and B16 cells are in agreement with previous studies (Knight, Souberbielle et al. 1996; Peter, Mezzacasa et al. 2001).

Melanoma Antigen	B16	K1735
Tyr	+	-
TRP-1	+	-
TRP-2	+	+
gp100	+	+
Mage A1	-	-
Mage A2	+	+
Mage A4	+	+
Mage A7	-	-

Table 4.1: Analysis of melanoma associated antigen expression by K1735 and B16 cells.
Total RNA was isolated from cells, cDNA generated and expression of TRP-1, TRP-2, Tyr, gp100, MAGE-1, MAGE-2, or MAGE-3 analysed by PCR using specific primers for each of these molecules. GAPDH signal was used to confirm integrity of RNA. (+ = presence of appropriate band, - = absence of appropriate band).

4.3 Histological examination of injection sites following subcutaneous injection of irradiated K1735 cells

To investigate how vaccine preparations of K1735 cells are recognised by the host immune system, irradiated (100Gy) K1735 cells ($5x10^5$ cells/injection) were injected subcutaneously into the flank of C57BL/6 mice and the boundaries of the injection site, covering an area of approximately 1x1cm, were marked. At 0.5, 6, 24, 48, 72 and 96 hrs following injection animals were sacrificed, the injection site harvested and processed appropriately for histology. For generating frozen sections, the tissues were immersed in OCT medium immediately after harvesting and snap frozen. The frozen blocks of tissue were then sent to the Pathology Department (Mayo Clinic) and 5μm sections were cut and stained for H&E for general structural observations. Although repeated attempts were made, we were unable to obtain sections that maintained good tissue morphology. This was likely due to the mixed consistency of the harvested tissue, which contained both the dense skin layer and the softer subcutaneous space of the injection site. We were therefore unable to obtain frozen sections for staining with a variety of cell markers for different cell types, such as $CD4^+$ T cells, $CD8^+$ T cells, DC, Mϕ, or NK cells.

Maintenance of very good tissue morphology has been well documented with paraffin-embedded tissue (Gendelman, Moench et al. 1983; Whiteland, Nicholls et al. 1995), although often this is at the expense of preserving antigenic epitopes (Whiteland, Nicholls et al. 1995). As an alternative to frozen sectioning, paraffin-embedded sections were prepared. Tissue was harvested at 0.5, 24, 48 and 72 hrs following injection, fixed in 10% neutral buffered formalin immediately following harvesting for a minimum of 24hrs, paraffin embedded and sectioned (5μm thick sections). The tissue was then stained with H&E or with a variety of antibodies to different cell-surface markers. Specifically, antibodies against CD4, CD8, DEC-205 and CD11b were used to detect the presence of $CD4^+$ T cells, $CD8^+$ T cells, DC and Mϕ respectively. Although very good tissue morphology could be obtained, as determined by H&E staining, no reproducible staining could be achieved using any of the above-mentioned antibodies in any of the conditions attempted. This is consistent with literature reports that in formalin-fixed, paraffin-embedded tissue, critical

antigenic epitopes are very commonly lost, and that successful staining for immune cells could be obtained only under very specialized fixing, embedding, and processing conditions (McLean and Nakane 1974; Collings, Poulter et al. 1984; Brenes, Harris et al. 1986; Whiteland, Nicholls et al. 1995).

Nonetheless, careful examination of the H&E stained sections revealed meaningful information about the local inflammatory environment generated at the site of injection of the allogeneic K1735 vaccine. Analysis was performed on the basis of morphology of the cells as revealed by H&E. **Figure 4.2** shows that irradiated K1735 cells attracted an increasingly larger mononuclear cell infiltrate as time progressed. Furthermore, analysis of B16 injection sites showed that irradiated B16 cells attracted an immune infiltrate also, although at a less marked level compared to K1735 cells. PBS injection site showed the basal levels of immune cells at the injection sites at the different timepoints.

4.4 Allogeneic K1735 cells are progressively cleared from the injection site following subcutaneous injection and stimulate a pro-inflammatory cytokine response

Irradiated (100Gy) K1735-Neo cells were injected subcutaneously as described above and the injection site was harvested at 0.5, 24, 48, 72 and 96 hrs following injection. Total RNA was extracted from the tissue, cDNA generated and used as a template for an PCR reaction for the detection of the Neo marker gene in the harvested tissues. This was an indirect method of determining the rate of clearance of the allogeneic K1735 cells by the host immune system. As it can be seen in Figure 4.3 the Neo signal disappeared by 72 hrs suggesting that the allogeneic cells were progressively cleared from the injection site within a timeframe of approximately 72 hrs. In addition to determining the presence of the allogeneic cells, the injection sites were analyzed for expression of a panel of pro- and anti-inflammatory cytokines. Primers specific for murine IL-10, TNF-α and INF-γ were designed based on the published sequence for these molecules. rtPCR analysis revealed that a significant pro-inflamatory cytokine response was induced at the subcutaneous injection site against the K1735-Neo cells, with induction of TNF-α and INF-γ at 24-96 hrs following vaccination. No induction of IL-10 was observed up to 4 days following vaccination.

Induction of pro-inflammatory cytokine expression correlated well with the time course of clearance of the allogeneic cells from the site of injection, suggesting that activated infiltrating immune cells are mediating the clearance of the vaccine cells.

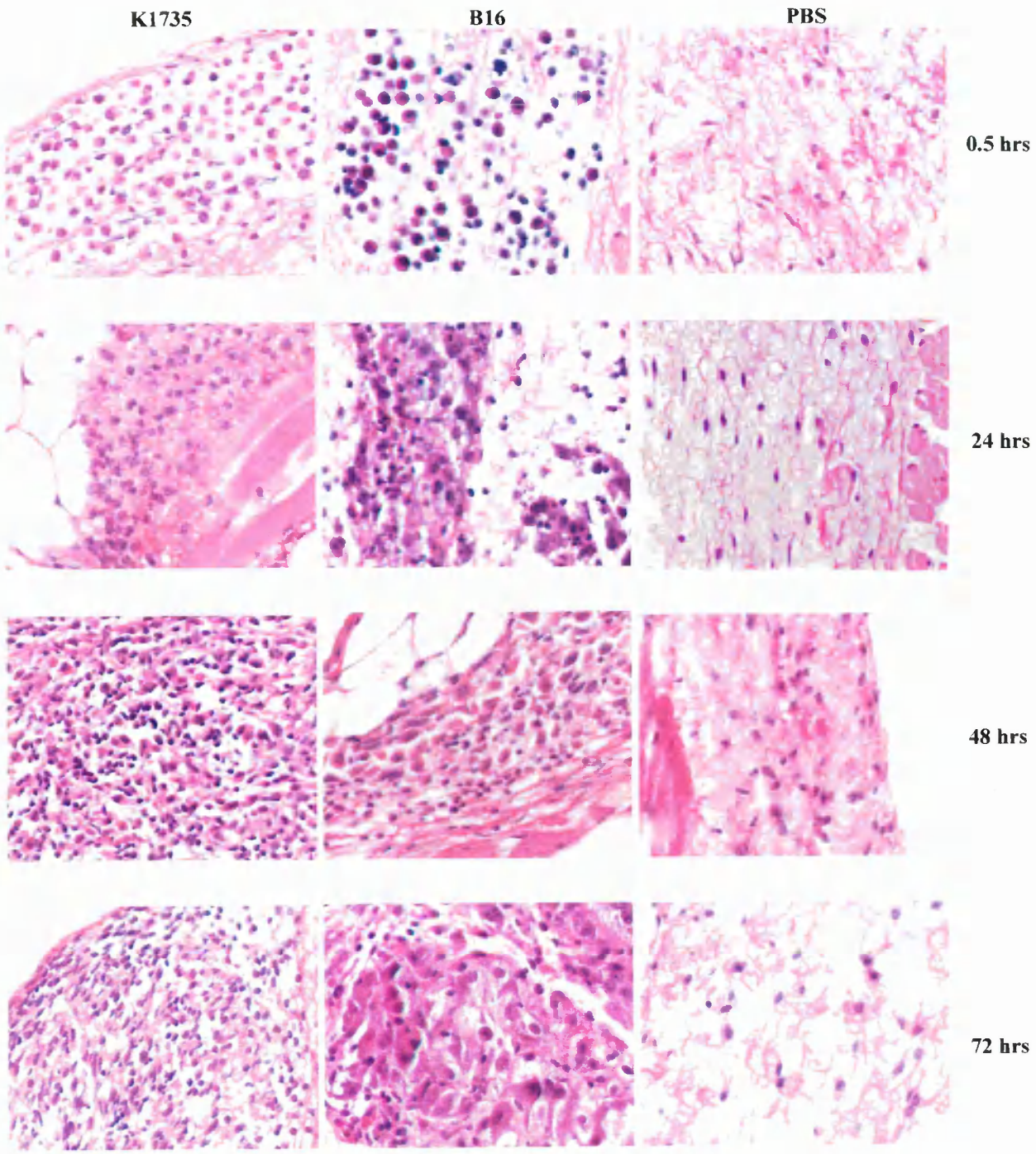


Figure 4.2: Histological analysis of injection sites.
$5x10^5$ irradiated (100Gy) K1735 or B16 cells or PBS were injected subcutaneously into the flank of mice. At 0.5, 24, 48 and 72 hrs the injection site was harvested, fixed in 10% neutral buffer formalin and paraffin-embedded. 5μm sections were cut from each tissue and stained for H&E (Magnification: 63X).

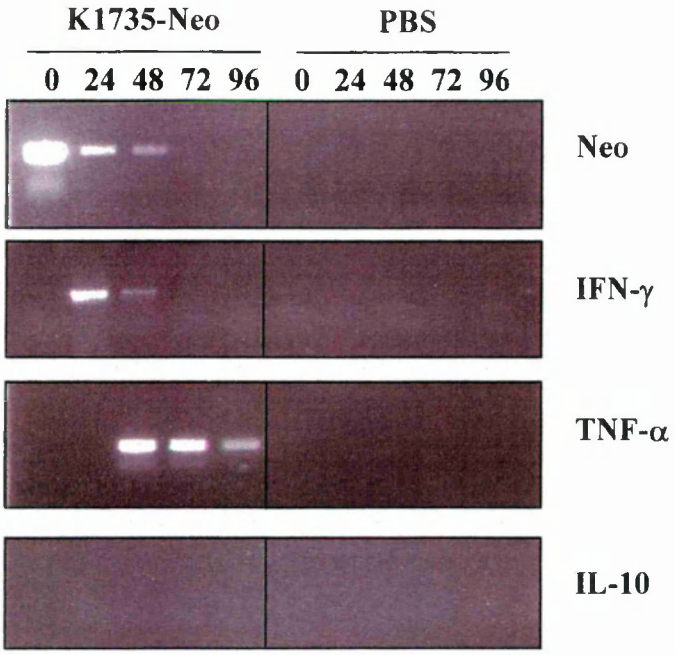


Figure 4.3: Subcutaneous vaccination with allogeneic K1735 cells induces local expression of pro-inflammatory cytokines.
RNA recovered from vaccination sites was used for rtPCR to screen for expression of murine cytokines at 0.5, 24, 48, 72 or 96 hrs following vaccination with $5x10^5$ irradiated K1735-neo cells or PBS. Equal loading of RNA was assessed by expression of GAPDH (data not shown).

4.5 K1735 vaccination induces infiltration of NK cells, Mϕ, and granulocytes

The histological and rtPCR analyses strongly suggested that irradiated K1735 cells can attract immune cells at the site of injection and can be cleared in an immunostimulatory fashion. Since our attempts to identify the profile of immune infiltrates at the injection site with immunostaining of histological sections of the injection site were unsuccessful, we designed an indirect method of studying the infiltrates (**Figure 4.4**). Gelfoam absorbable gelatin sponges were surgically implanted into the subcutaneous space on the backs of mice (two sponges of 1.5x1.5x0.5cm each were implanted per mouse) and the animals were allowed to recover from the procedure for a period of three days. At day three, $5x10^5$ irradiated (100Gy) K1735 or B16 cells were injected directly into the sponges and 48 hrs later the sponges were surgically removed for analysis. Sponges were treated with collagenase for 3 hrs to dissolve them and release any infiltrating cells. Red blood cells were depleted, cells counted and phenotyped by FACS analysis using a combination of antibodies to specific cell surface markers of immune cells. To

determine the basal levels of infiltrating cells in this system, sponges from animals that received a PBS injection were also analysed in each experiment.

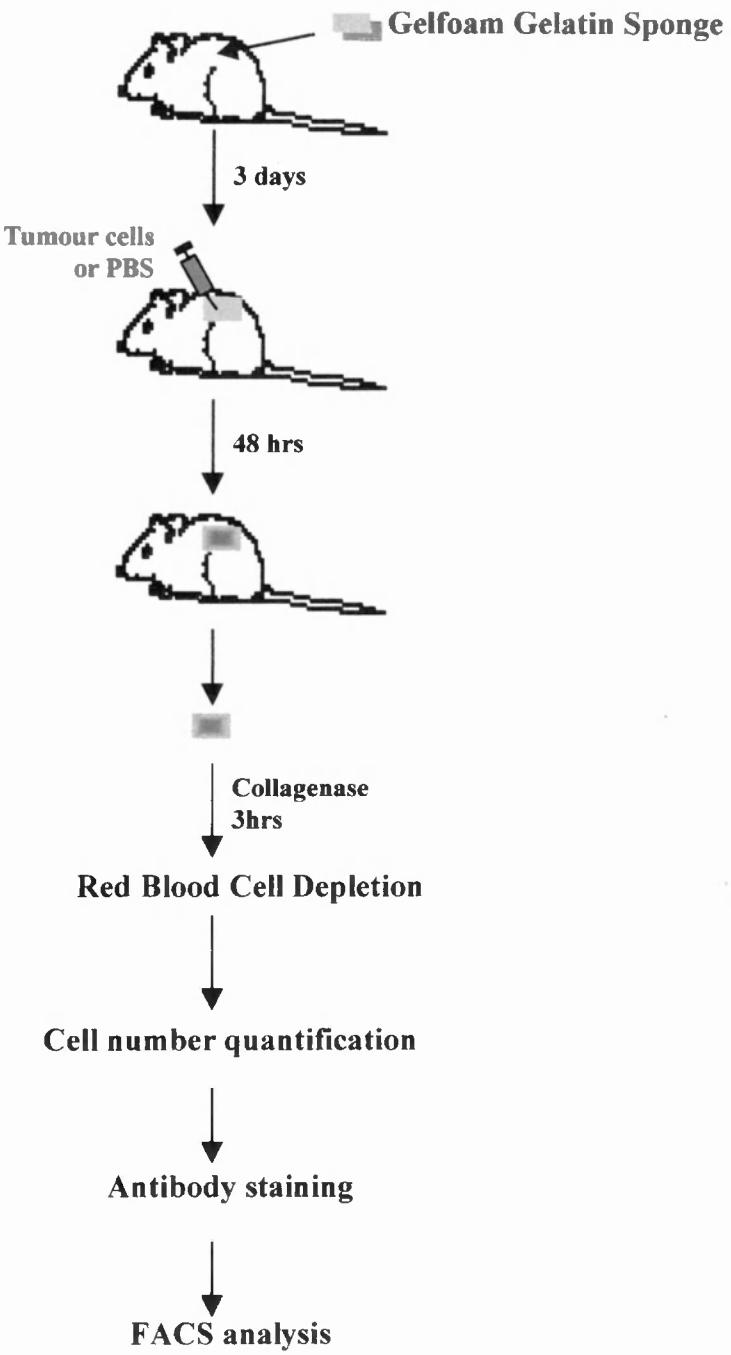
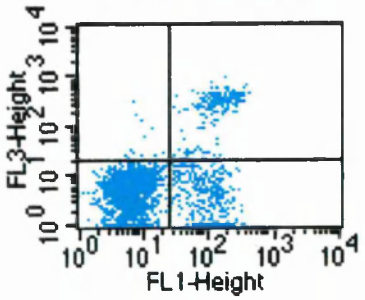


Figure 4.4: Diagrammatic description of sponge experiments in mice.
Gelfoam absorbable gelatin sponges of approximately 1x1x0.5cm were surgically implanted at the back of animals. 3 days later tumour cells or PBS were injected into sponges and 48 hrs later sponges were harvested and digested with collagenase to release cells from sponge matrix. Red blood cells were removed and cells quantified, stained with a panel of cell surface markers and analysed by FACS.

Figure 4.5 shows a typical FACS dot plot and the statistical analysis of the results. The final number of each specific infiltrating cell type was calculated by multiplying the percentage total positive-staining cells by the total number of cells harvested from the sponge. Numbers of CD8 T cells ($CD8^+/CD3^+$), CD4 T cells ($CD4^+/CD3^+$), NK cells ($NK1.1^+/CD3^-$), macrophages ($Mac3^+/CD11c^-$), DCs ($CD11c^+/I\text{-}A^{b+}$), granulocytes ($Ly6G^+/CD14^-$) and total leukocytes ($CD45^+$) were determined by costaining and multicolor FACS analysis. Irradiated K1735 vaccine cells injected into a sponge attracted a significantly higher number of NK cells, Mϕ and granulocytes (**Figure 4.6**) compared to the PBS control, confirming that irradiated K1735 cells can are recognised by the host immune system under these conditions. In addition, $CD8^+$ and $CD4^+$ T cell, and DC infiltration was increased. The autologous B16 cells attracted an increased level of leukocytes compared to the PBS controls. Infiltration observed in the K1735 sponges was consistently higher compared to that observed in the B16 sponges for all the cells types examined. These data are consistent with the histological analysis showing immune infiltration of the injection site, as well as the cytokine analysis showing the generation of a pro-inflammatory environment.

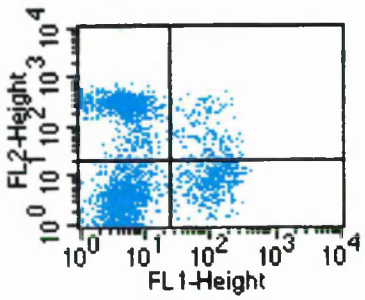
CD4-FITC/**CD3**-PerCP



Gate: G4
Gated Events: 2194
Total Events: 40000

Quad	Events	% Gated	% Total
UL	14	0.64	0.03
UR	210	9.57	0.53
LL	1672	76.21	4.18
LR	298	13.58	0.74

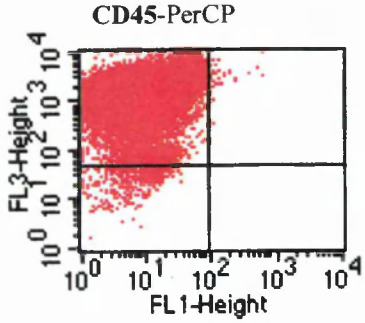
CD3-FITC/**NK1.1**-PE



Gate: G4
Gated Events: 2225
Total Events: 40000

Quad	Events	% Gated	% Total
UL	754	33.89	1.88
UR	145	6.52	0.36
LL	995	44.72	2.49
LR	331	14.88	0.83

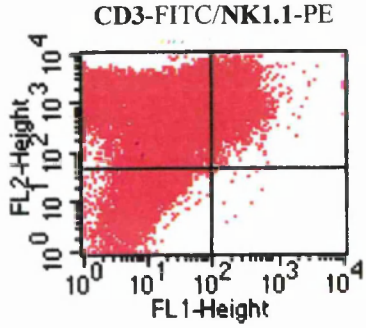
CD45-PerCP



Gate: G1
Gated Events: 37000
Total Events: 40000

Quad	Events	% Gated	% Total
UL	36368	98.29	90.92
UR	36	0.10	0.09
LL	596	1.61	1.49
LR	0	0.00	0.00

CD3-FITC/**NK1.1**-PE



Gate: G1
Gated Events: 36944
Total Events: 40000

Quad	Events	% Gated	% Total
UL	26131	70.73	65.33
UR	4422	11.97	11.05
LL	6380	17.27	15.95
LR	11	0.03	0.03

Figure 4.5: Dot plots and statistical analysis of sponge infiltrating cells.
Cells extracted from the sponges were stained with antibodies to surface molecules conjugated to FITC (FL-1), PE (FL-2) or PerCP (FL-3), and analysed using FACS. Typical dot plots and corresponding statistical analysis are shown.

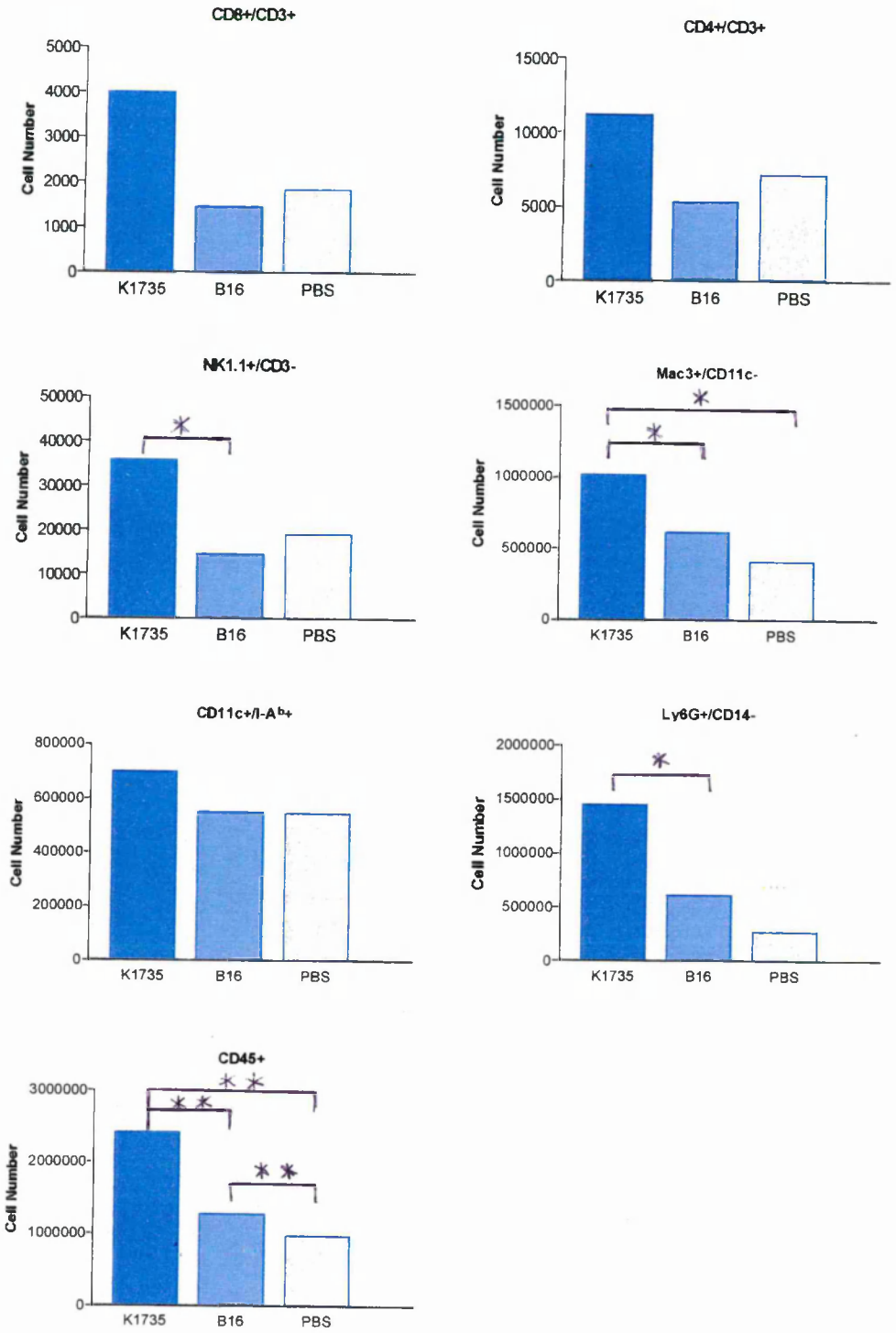


Figure 4.6: Irradiated K1735 cells attract immune cells at site of injection.
K1735 or B16 or PBS was injected into implanted sponges in mice and 48 hrs later the sponges were harvested and infiltrating cells were analysed as described in Figure 4.3 and Figure 4.4. To calculate number of infiltrating cells in each sponge,% total cells obtained for each sponge analysed were multiplied by the total number of cells harvested from that sponge. The trends shown here are representative of three individual experiments (* $P<0.05$; ** $P<0.01$).

4.6 Vaccines consisting of mixtures of allogeneic K1735 and syngeneic B16 cells have no significant protective effect against a B16 challenge

The data thus far have shown that K1735 cells express allogeneic MHC class I on their surface, express some melanoma associated antigens and can attract immune cells at a subcutaneus injection site and stimulate a pro-inflammatory immune response. Syngeneic B16 cells express no detectable MHC class I on their surface, express a wide range of melanoma associated antigens tested and can attract some immune infiltrate at the injection site. Based on this information, we decided to investigate what would be the effects of providing relevant tumor antigens to an allogeneic vaccine, by adding autologous cells to the vaccine. Alternatively, what would be the effects of providing allogenicity to a syngeneic vaccine, by adding allogeneic cells to the vaccine.

Mice received three subcutaneous consecutive injections of $5x10^5$ 100Gy-irradiated K1735 cells or $5x10^5$ 100Gy-irradiated B16 cells or different mixtures of the two cell lines as shown in **Figure 4.7**. Survival of animals following a B16 tumour challenge was monitored. The results shown in Figure 4.7 are from a single experiment, representative of three separate experiments, and for purposes of clarity they were separated into two individual graphs, A and B. Addition of increasing numbers of autologous B16 cells had no significant effect on the immunogenicity of the K1735 cells against a B16 challenge (**Figure 4.7.A**). Likewise, addition of increasing numbers of allogeneic cells had no significant effect on the immunogenicity of the B16 cells against a B16 challenge (**Figure 4.7.B**). These data suggest that providing TAA with an allogeneic K1735 vaccine or providing potentially immunstimulatory molecules, such as the allogeneic MHC, with an autologous B16 vaccine has no effect on the immunogenicity of the allogeneic or the syngeneic vaccine respectively in the model used.

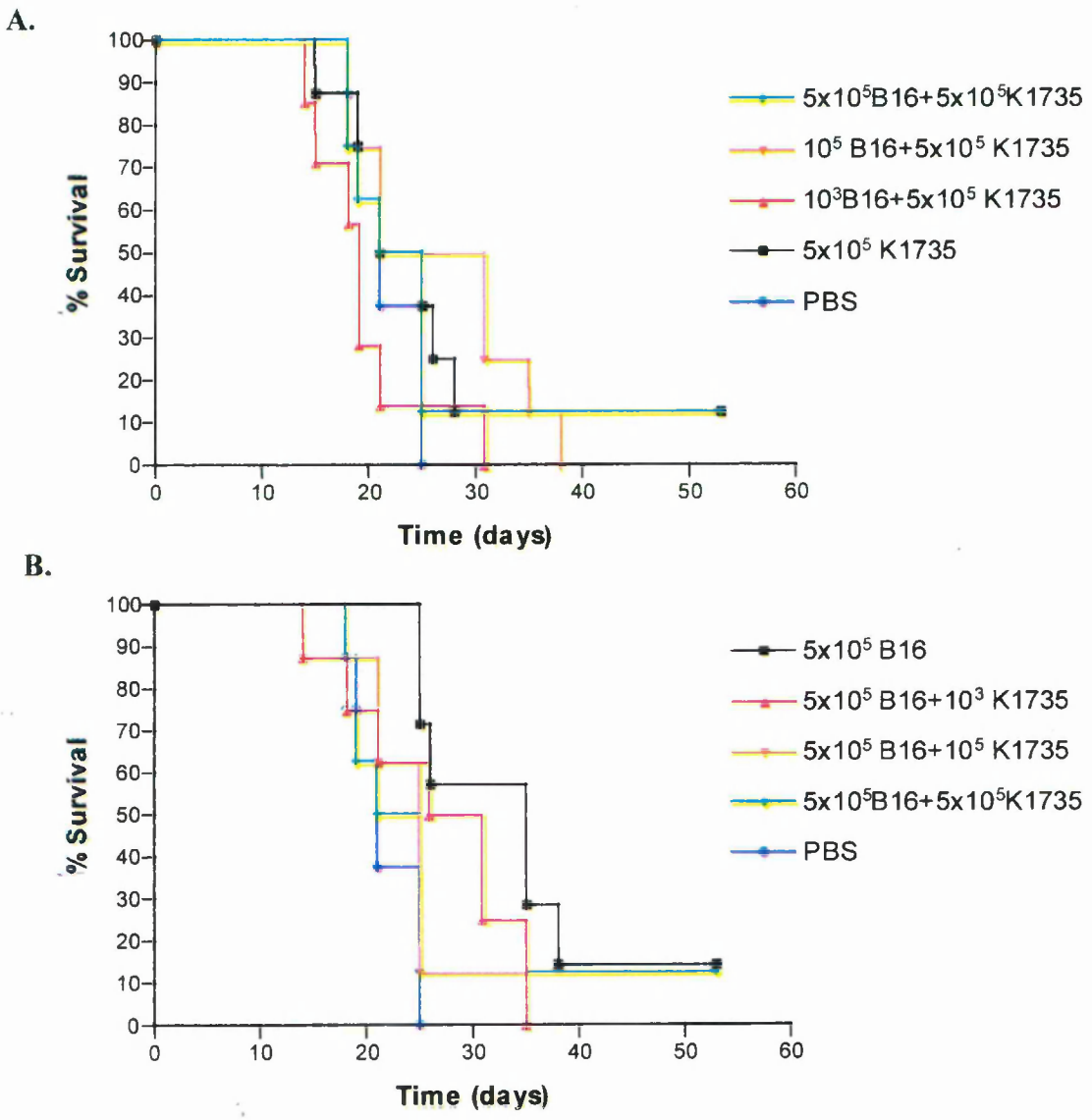


Figure 4.7: Semi allogeneic vaccines do not protect mice from a B16 challenge. Mice received three consecutive vaccination, 7 days apart, of $5x10^5$ irradiated K1735 cells or different ratios of irradiated K1735 and B16 cells or PBS. Seven days following the last vaccination mice were challenged with $2x10^5$ live B16 cells and the percentage of mice surviving following the challenge are shown (8 mice per group). For clarity, the separate groups of a single experiment are shown in the two individual graphs (A. and B.). (P=0.0018 $5x10^5$B16 vs PBS; P=0.0198 $5x10^5$B16 vs $5x10^5$B16+10^5K1735).

4.7 Summary

Allogeneic K1735 cells are MHC Class I positive, Class II negative, ICAM-I negative, B7.1 low expressing and B7.2 negative. They express some melanoma associated antigens, such as TRP-2, gp100, MAGE-2 and MAGE-7 and irradiated cells can attract immune cells at a subcutaneous injection site. B16 cells express no MHC Class I or II molecules on the cell surface, and no B7.1, B7.2 or ICAM-I. They express a wide panel of melanoma associated antigens, including TRP-1, TRP-2, Tyr, gp100, MAGE-2, and MAGE-4. Sponge experiments revealed that irradiated allogeneic K1735 cells attract higher levels of NK cells, Mϕ, granulocytes, $CD8^+$ and $CD4^+$ T cells at the injection site when compared to B16 or PBS control injection sites. To address the issue of whether the weak immunogenicity of the allogeneic K1735 vaccine could be attributed to a relative paucity of TAA expressed by the vaccine cells, mice were vaccinated with different mixtures of K1735 and B16 cells. The immunogenicity of the allogeneic K1735 vaccine could not be enhanced when different amounts of B16 cells were mixed in the vaccine. Furthermore, the immunogenicity of a B16 vaccine could not be enhanced when different levels of K1735 cells were mixed in the autologous vaccine.

These data suggest that K1735 cells can be immunostimulatory when injected as a whole tumor cell vaccine, but the immune response generated against such a vaccine is not appropriate or sufficient to generate a systemic protective immunity against a syngeneic melanoma challenge. Modification of the allogeneic K1735 vaccine to express potentially immunostimulatory molecules, such as IL-12, GM-CSF, hsp70 and INF-γ (Chapter 3), does not enhance the immunogenicity of the allogeneic vaccine any further.

Chapter 5

VSVG-G-Mediated Fusion Enhances immunotherapeutic Potential of a Semi-Allogeneic Vaccine

Allogeneic K1735 melanoma cells, although they can not vaccinate against a syngeneic B16 challenge, can induce a local proinflamatory environment and enhance the infiltrate of immune cells at the site of vaccination. We aimed to enhance this effect by controlling the destruction of the allogeneic cells in the vaccination site via expression of cytotoxic genes. We have previously shown that expression of viral FMG genes in tumour cells leads to very potent local tumor cell killing through fusion of tumor cells to each other (Bateman, Bullough et al. 2000; Diaz, Bateman et al. 2000; Fielding, Chapel-Fernandes et al. 2000). The fusion event leads to the formation of large multinucleated syncytia which subsequently die through non-apoptotic, autophagic-like mechanisms which are immune potentiating (Suhy, Giddings et al. 2000; Bateman, Harrington et al. 2002). In addition, death of syncytia is associated with the induction of stress related proteins (Bateman, Bullough et al. 2000) which are potently immunostimulatory (Melcher, Todryk et al. 1998; Gough, Melcher et al. 2001). Finally, we (Bateman, Bullough et al. 2000) and others, have observed that the expression of viral immunogens can act as potent adjuvants in vaccinating against tumor related antigens through various immunological mechanisms (Schirrmacher, Haas et al. 1999; Eslahi, Muller et al. 2001; Mandelboim, Lieberman et al. 2001). Therefore, we reasoned that using FMG to fuse tumor cells to each other along with the immune stimulatory mechanisms associated with FMG-mediated cell killing may provide an effective method to liberate relevant tumour antigens from vaccine cells in an immunologically effective context.

5.1 Transient expression of the gene for Vesicular Stomatitis Virus G glycoprotein leads to the formation of extensive syncytia

We used transfection of the gene for VSV-G to fuse murine tumor cells to each other. VSV-G is non-fusogenic until the ambient pH is lowered to pH 5.5-5.7, which is believed to induce a conformational change that converts the protein into a fusogenic conformation(Fredericksen and Whitt 1995; Han, Bushweller et al. 2001). Since expression of the VSV-G envelope protein may be a potent adjuvant of itself (in the

absence of tumor cell fusion) (Schirrmacher, Haas et al. 1999; Bateman, Bullough et al. 2000; Eslahi, Muller et al. 2001; Mandelboim, Lieberman et al. 2001), we also used a mutant VSV-G in which a single amino acid mutation at position 124 of the protein reduces the amount of cell fusion induced by expression of the gene by over 90% (Fredericksen and Whitt 1995). Transfection with this mutant VSV-G also served as a control for the transient pH drop to which all fusing vaccine preparations were exposed. Transfection of tumour cells with VSV-G followed 24 hours later by a 2 minute drop in pH to 5.5-5.7 caused fusion of cells such that between 60-90% of the cells *in vitro* were involved in syncytia 24-48 hours following the pH drop (**Figure 5.1**). Transfection with VSV-G G-E124 or mock transfection routinely fused minimal numbers of cells (**Figure 5.1** and (Fredericksen and Whitt 1995)).

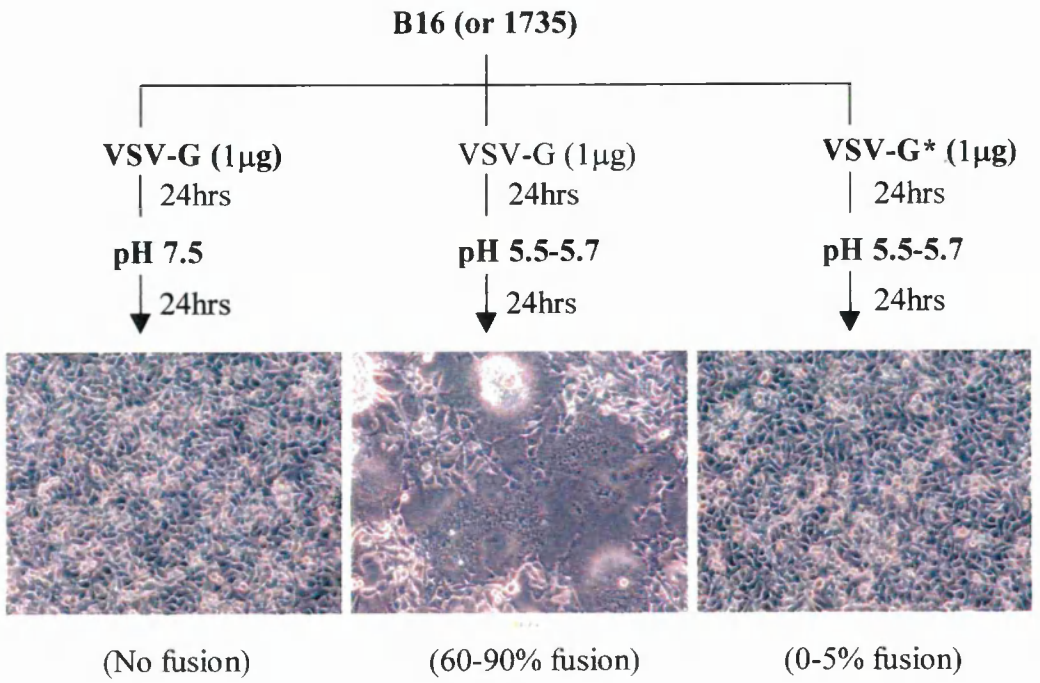


Figure 5.1: Extensive tumour cell fusion mediated by VSV-G expression and low pH acid shock.
B16 tumour cells transfected with VSV-G or the VSV-G G-E124 mutant (VSV-G*). 24 hours later cells were either left in normal pH media (pH 7.5) or briefly exposed to media of pH 5.7 for 2 min. 24-48 hours later cells transfected with VSV-G and acid shocked formed large multinucleated syncytia. In constrast cells transfected with VSV-G but not exposed to low pH or cells transfected with VSV-G* showed minimal amounts of fusion.

5.2 VSV-G mediated tumour cell fusion does not enhance the efficacy of an autologous or an allogeneic tumour cell vaccine

We tested the effects of vaccination of C57/BL mice with VSV-G-mediated fusing cell vaccines in the prophylactic protection model previously described in chapters 3 and 4. In one experiment, fusion of B16 cells to each other with VSV-G generated a moderately more effective vaccine compared to an irradiated B16 whole tumour cell vaccine, which is non-immunogenic in the experimental conditions used in these studies, or no vaccination (**Figure 5.2A**). In the same experiment, transfection of B16 with the non-fusing VSV-G, had a similar effect to the fusing B16 vaccine (**Figure 5.2A**), suggesting that expression of the VSV-G envelope protein in B16 cells slightly enhances their immunogenicity. However, to show any statistical significance of the observed effect, an analysis of much larger experimental group sizes would have to be done over several experiments. Fusion of the allogeneic K1735 cells to each other by VSV-G had no effect on the immunogenicity of the vaccine compared to naïve (untreated) animals (**Figure 5.2B**). Similarly, expression of VSV-G in the absence of fusion had no effect on the immunogenicity of the K1735 (**Figure 5.2B**).

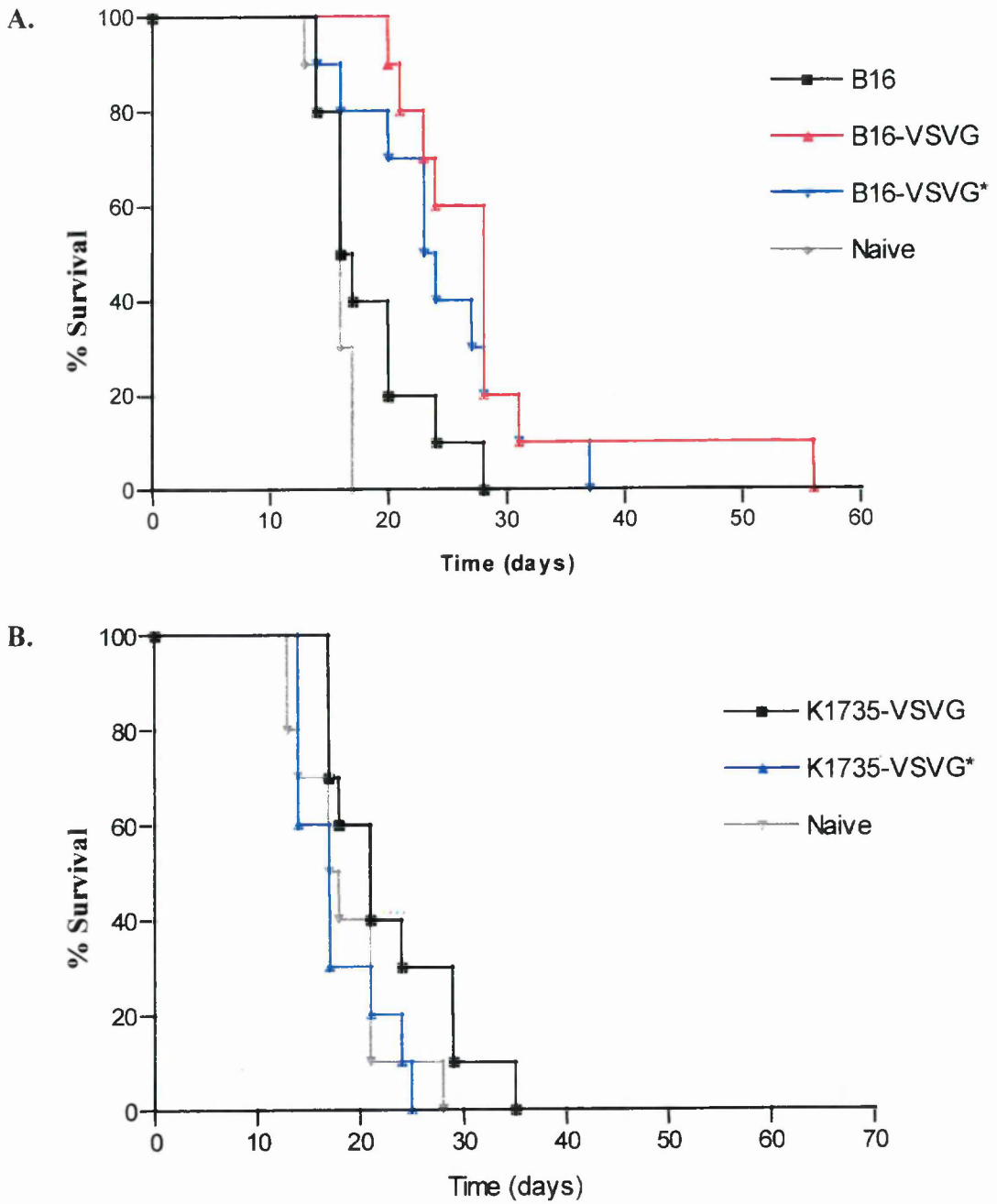


Figure 5.2: Irradiated fusing allogeneic or autologous vaccines are very poor immunogens against a B16 challenge.

Groups of 10 C57BL/6 mice received 3 sequential subcutaneous injections of irradiated vaccine cells followed by a live B16 challenge. The percentage of mice surviving are shown following the B16 challenge. Mice were sacrificed once tumour size exceeded 1cm in diameter. **A.** vaccine cells: VSV-G transfected fusing B16 (B16-VSVG); VSV-G G-E124 transfected non-fusing B16 (B16-VSVG*); parental B16 (B16); no vaccination (Naïve). **B.** vaccine cells: VSV-G transfected fusing K1735 (K1735-VSVG); VSV-G G-E124 transfected non-fusing K1735 (K1735-VSVG*); no vaccination (Naïve).

5.3 VSV-G mediated tumour cell fusion enhances the efficacy of autologous/allogeneic tumour cell vaccines

The data so far suggested that a purely autologous or a purely allogeneic fusing vaccine had no significant therapeutic effect against a B16 melanoma challenge in our protection model. Next we decided to investigate if a mixed allogeneic/autologous fusing vaccine would have a different effect. We hypothesized that the autologous cells would provide all the immunologically relevant antigens, the allogeneic cells would provide a strong adjuvant effect and the VSV-G mediated cell death would be a immunostimulatory mechanism of release of antigens accompanied by the induction and release of stress and danger signals. **Figure 5.3** shows that fusion of a 1:1 mixture of K1735:B16 cells with VSV-G generated a very effective vaccine with up to 70% of mice surviving rechallenge long term (>60 days) depending upon the individual experiment (the therapeutic effect ranged from 30% to 70%). These effects were not due to the adjuvant effect of the VSV-G immunogen alone, nor were they due to vector related components or to the brief exposure of cells to low pH, since the fusion defective mutant VSV-G G-E124 (VSV-G*) gave only minimal therapeutic gains (not statistically significant).

To understand the immunological significance of the mechanism by which the tumour cells are fused, the prophylactic vaccination experiments of Figure 5.3 were also carried out by fusing the K1735:B16 cells using polyethylene glycol (PEG) (Wang, Saffold et al. 1998). PEG fusion of tumour cells leads to the formation of disorganised cell aggregates rather than organised fusing cell monolayers obtained with VSV-G induced fusion. PEG-mediated fusion of tumour cells gave no delay in the appearance of tumours on rechallenge compared to controls, whereas fusion induced by VSV-G protected some mice long term from tumour growth (**Figure 5.4**). This indicates that the mechanism of VSV-G mediated fusion is crucial to the immunogenicity of fusing tumour cell vaccines.

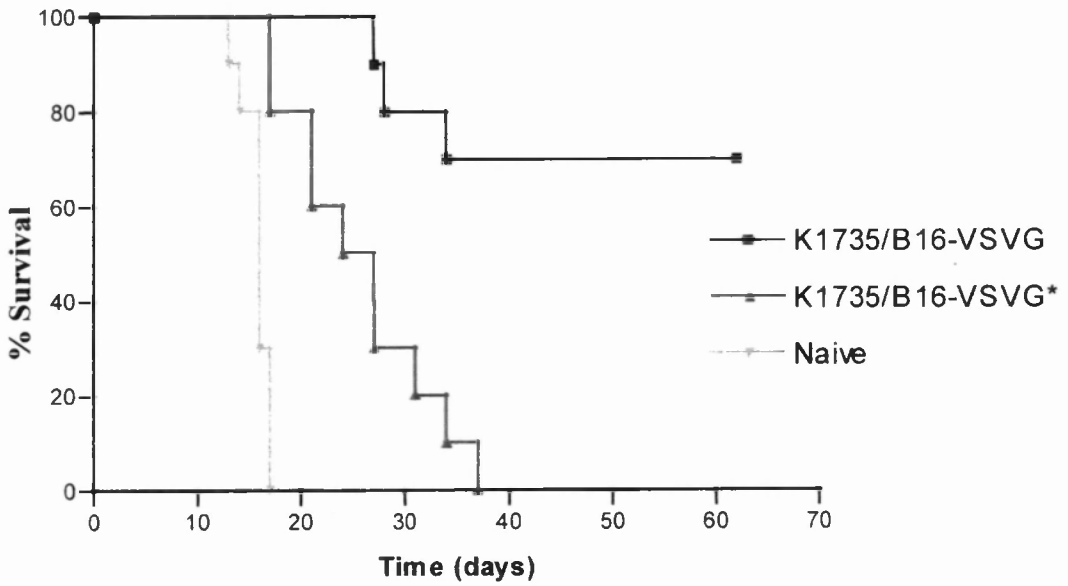


Figure 5.3: Irradiated fusing allogeneic/autologous vaccines are potent immunogens against B16 challenge.
Groups of 10 C57BL/6 mice received 3 sequential subcutaneous injections of a 1:1 mixture of irradiated K1735 and B16 cells transfected with VSV-G and fusing (K1735/B16-VSVG), or transfected with VSV-G*, non-fusing (K1735/B16-VSVG*), followed by a live B16 challenge. The percentage of mice surviving are shown following the B16 challenge. Mice were sacrificed once tumour size exceeded 1cm in diameter. Non vaccinated, naïve mice are shown as control. K1735/B16-VSVG was significantly better than K1735/B16-VSVG* or naïve groups, $P<0.0003$.

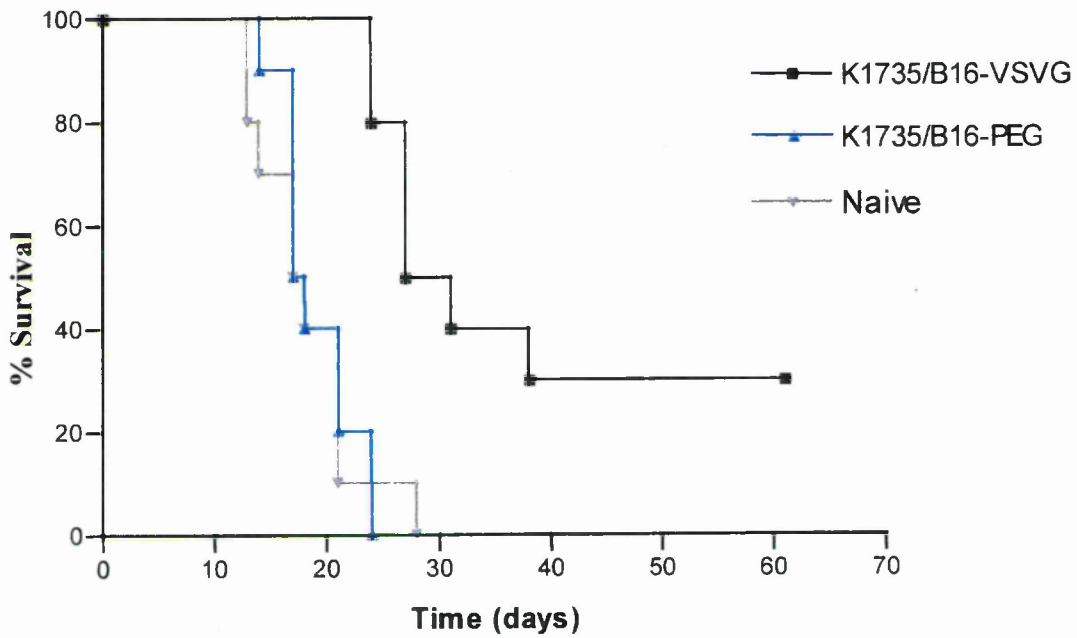


Figure 5.4: The mechanism of fusion is critical for vaccination efficacy.
A 1:1 mixture of K1735 and B16 cells fused by VSV-G (K1735/B16-VSV-G) or by polyethelene glycol (PEG) (K1735/B16-PEG) were used as irradiated cell vaccines in the prophylactic vaccination model similar to those described in Figures 5.2 and 5.3. The percentage of mice surviving are shown following the B16 challenge. Mice were sacrificed once tumour size exceeded 1cm in diameter. Non vaccinated, naïve mice are shown as control. K1735/B16-VSVG was significantly better than the K1735/B16-PEG ($P<0.0001$) or naïve ($P=0.0002$) groups.

5.4 Fusing tumor cell vaccines are effective in a therapy model of disease

We also tested the fusing tumour cell vaccines in a model of established disease (Castleden, Chong et al. 1997). Mice were seeded with B16 tumours on one flank, then treated with three consecutive vaccine treatments, 24 hours apart, on the contralateral flank once the B16 tumours became palpable (tumour diameter ~ 0.1cm), typically 3-5 days after seeding. Fusing B16:K1735 vaccines were able to cure up to 80% of mice with established disease in this model (**Figure 5.5**). Both K1735:K1735 and B16:B16 fusing vaccines consistently produced a significant delay in growth of the tumour and, depending on the experiment, cured between 20-40% of the mice (**Figure 5.5A**). Other vaccines produced no therapeutic effect (**Figure 5.5**).

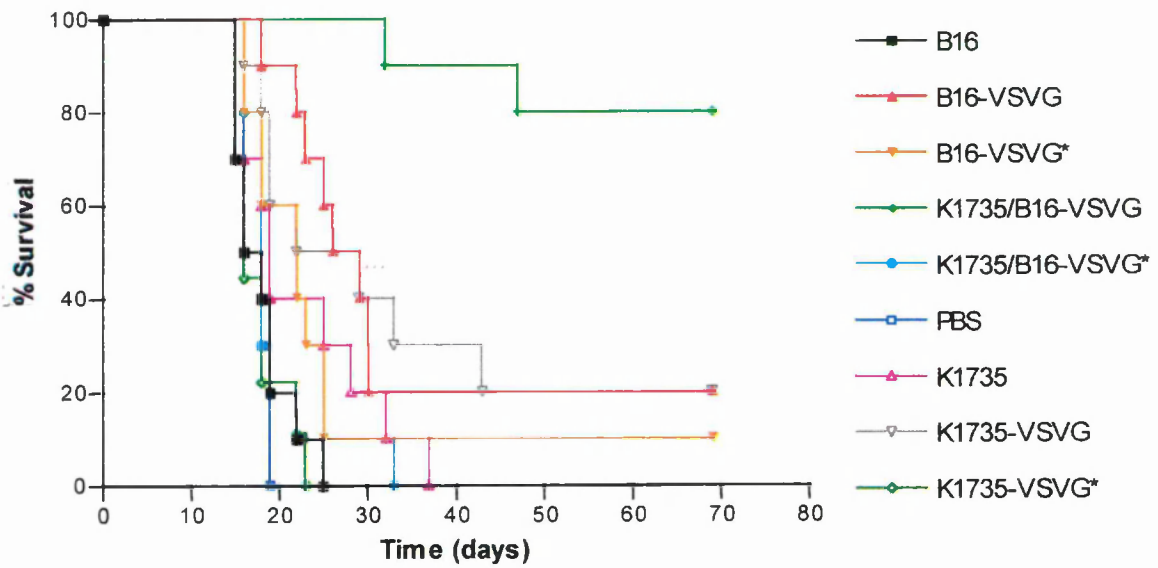


Figure 5.5: Fusing allogeneic/autologous cells are potent immunogens against established disease.
Fusing cell vaccines were tested in a therapy model. Groups of 10 C57BL/6 mice were injected with $2x10^5$ live B16 cells. Once the B16 tumours became palpable, the mice received three vaccines separated by 24 hours. The percentage of mice surviving the lethal tumour challenge following treatment with different irradiated fusing (VSVG) or non-fusing (VSVG*) cell vaccines is shown post initial B16. Mice were sacrificed once tumour size exceeded 1cm in diameter. The K1735/B16-VSVG group was significantly better than any of the other groups ($P<0.0001$).

5.5 The long term vaccinating capability of fusing tumour cell vaccines is both tumour specific and T cell dependent

In the experiment shown in Figure 5.5, we rechallenged the 8 mice which had been cured of their tumours by treatment with K1735:B16 fusing tumour vaccines. Of the 4 mice rechallenged with CMT93 tumour cells – an unrelated colorectal tumour syngeneic with C57/BL mice – all 4 developed tumours (Table 5.1). In contrast, only 1 of 4 mice rechallenged with parental B16 cells developed a tumour (Table 5.1). These data show that the immunological mechanisms that led to effective rejection of the established B16 tumours by the K1735:B16 fusing vaccine were able to contribute significantly to the development of long term immunity against the tumour. Moreover, this long term protective effect is tumour specific.

Initial vaccination	Survival	Rechallenge	Survival 25 days post rechallenge
K1735/B16-VSVG	8/10	B16 (4/8)	3/4
		CMT (4/8)	0/4

Table 5.1: Tumour specific, long term vaccinating capability of fusing tumour cell vaccines.
In the experiment shown in Figure 5.5, 8 of 10 mice were cured of the established tumours when treated with a fusing K1735/B16 vaccine. These 8 mice were rechallenged with either parental B16 or parental CMT93 colorectal tumour cells. 25 days later, all the animals challenged with CMT93 cells had succumbed to progressively growing tumours. Only 1 of the 4 mice rechallenged with B16 developed a tumour and was sacrificed.

We also repeated the vaccination protocol of **Figure 5.3** in athymic nude mice. **Figure 5.6** shows that the vaccinating ability of the fusing cell vaccines was completely eradicated in animals lacking functional T cells, suggesting that T cells play an important role in the immune response mounted against the autologous B16 cells.

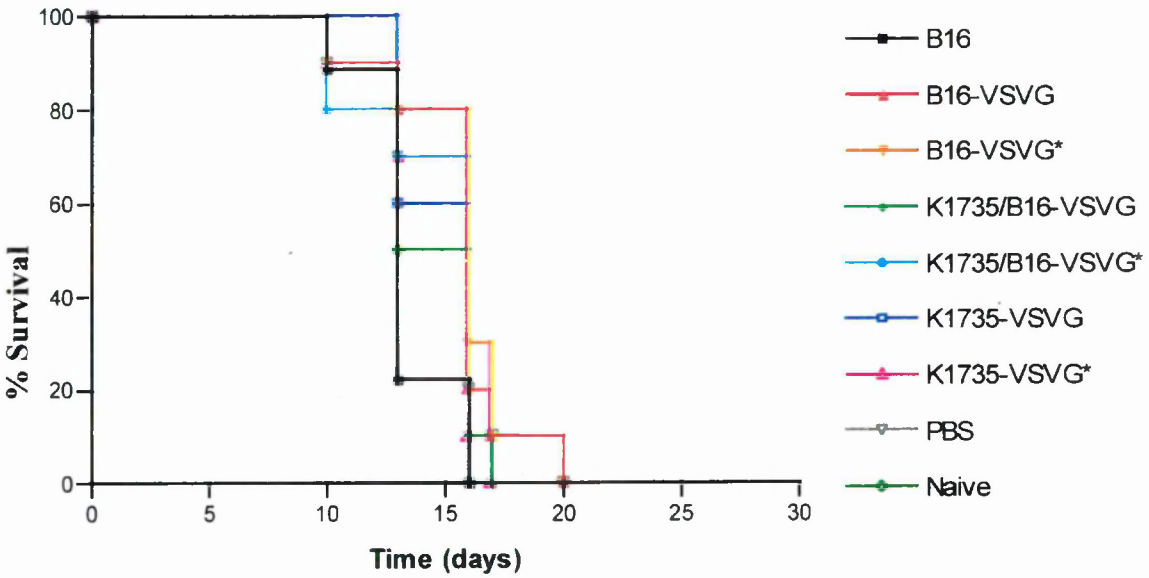


Figure 5.6: The long term vaccinating capability of fusing tumour cell vaccines is T cell dependant.
The prophylactic vaccination experiments of Figures 5.2 & 5.3 were repeated in athymic nude mice which lack T cells. As previously, mice were vaccinated with irradiated fusing (VSVG) or non-fusing (VSVG*) vaccines, PBS or no vaccine at all. The percentage of mice surviving are shown following the lethal B16 challenge. Mice were sacrificed once tumour size exceeded 1cm in diameter. There was no statistically significant difference between any of the groups.

5.6 Summary

Despite improvements in slowing tumour development in some cases, neither pure autologous nor pure allogeneic fusing vaccines alone were able to protect animals against development of disease. However, an irradiated mixture of autologous and allogeneic tumour cells fused by VSV-G was extremely effective at protecting mice from subsequent rechallenge with B16 cells. Furthermore, a similar vaccine was also very potent at curing small established disease. This protective immunity was not attributable to the expression of the immunogenic VSV-G alone, or to the possible effects of low pH exposure, since cells transfected with a mutant, non fusing VSV-G differing in only a single amino acid, were only weakly immunogenic. The protection effects observed here were long term, tumour specific and T cell dependent. Finally, fusion of a similar autologous/allogeneic vaccine by chemical means (polyetheline glycol-PEG) did not result in the generation of a potent immunogen, suggesting that the mechanism of fusion plays an instrumental role in the efficacy of a fusing vaccine.

It should be noted that, although the experiment in athymic nude mice is an indication on the possible importance of T cells in the immune response observed following the K1735/B16-VSVG fusing vaccination, these mice have a more complex immunocompromised phenotype that in not solely attributed to the absence of T cells. Hence, further experiments need to be done in models where the role of T cells can be examined in the absence of any other immunological defects, for example in CD4 or CD8 knockout animals or animals depleted of CD4+ or CD8+ T cell populations with specific antibodies. Along this line, experiments need to be done to investigate the role of non-specific immune effector cells such as NK cells in the potent immune response observed.

Chapter 6

MECHANISMS OF IMMUNOGENICITY OF FUSING TUMOUR CELL VACCINES

The data in Chapter 5 clearly demonstrate that expression of the VSV-G gene by a mixed autologous/allogeneic melanoma cell vaccine followed by fusion dramatically enhances the potency of such a vaccine. To understand the immunological mechanisms by which FMG-induced syncytial killing leads to the anti tumor effect we have observed in these studies we decided to study the host immune response against the fusing K1735/B16 vaccine, as well as the interaction of immune cells with the fusing vaccine *in vitro*.

6.1 VSV-G-mediated fusion enhances infiltration of immune cells at the site of vaccination.

We investigated the host response to the irradiated vaccine cells shortly after vaccination by utilising the gelfoam absorbable gelatine sponge model we had previously established (Chapter 4; Figure 4.4). K1735/B16-VSVG, K1735/B16-VSVG* and K1735/B16 vaccines were prepared as described in chapter 5, irradiated to 100Gy and injected into previously implanted gelfoam sponges at the flank of mice. 48 hrs later the sponges were removed from the animals and the cell infiltrates analysed. Numbers of CD8 T cells ($CD3^+CD8^+$), CD4 T cells ($CD3^+CD4^+$), NK cells ($CD3^-NK1.1^+$), Mϕ ($Mac3^+CD11c^-$), DC ($CD11c^+I\text{-}Ab^+$) and granulocytes ($Ly6G^+CD14^-$) were determined by costaining and multicolour FACS analysis, as previously described in Chapter 4, paragraph 4.5. **Figure 6.1** shows that expression of VSV-G* by the K1735/B16 vaccine did not significantly influence the profile of infiltrating cells when compared to the unmodified K1735/B16 vaccine. In contrast, K1735/B16-VSVG fusing cells consistently attracted significantly higher levels of NK cells, CD8 T cells, macrophages and granulocytes compared to K1735/B16 cells or K1735/B16-VSVG*. In only one of three experiments CD4 T cell infiltration was significantly higher in the fusing K1735/B16-VSVG group than the K1735/B16 or K1735/B16-VSVG* groups. The data shown in **Figure 6.1** are representative of at least three different experiments (except for $CD4^+CD3^+$ cells). VSVG expression and fusion did not seem to affect DC infiltration.

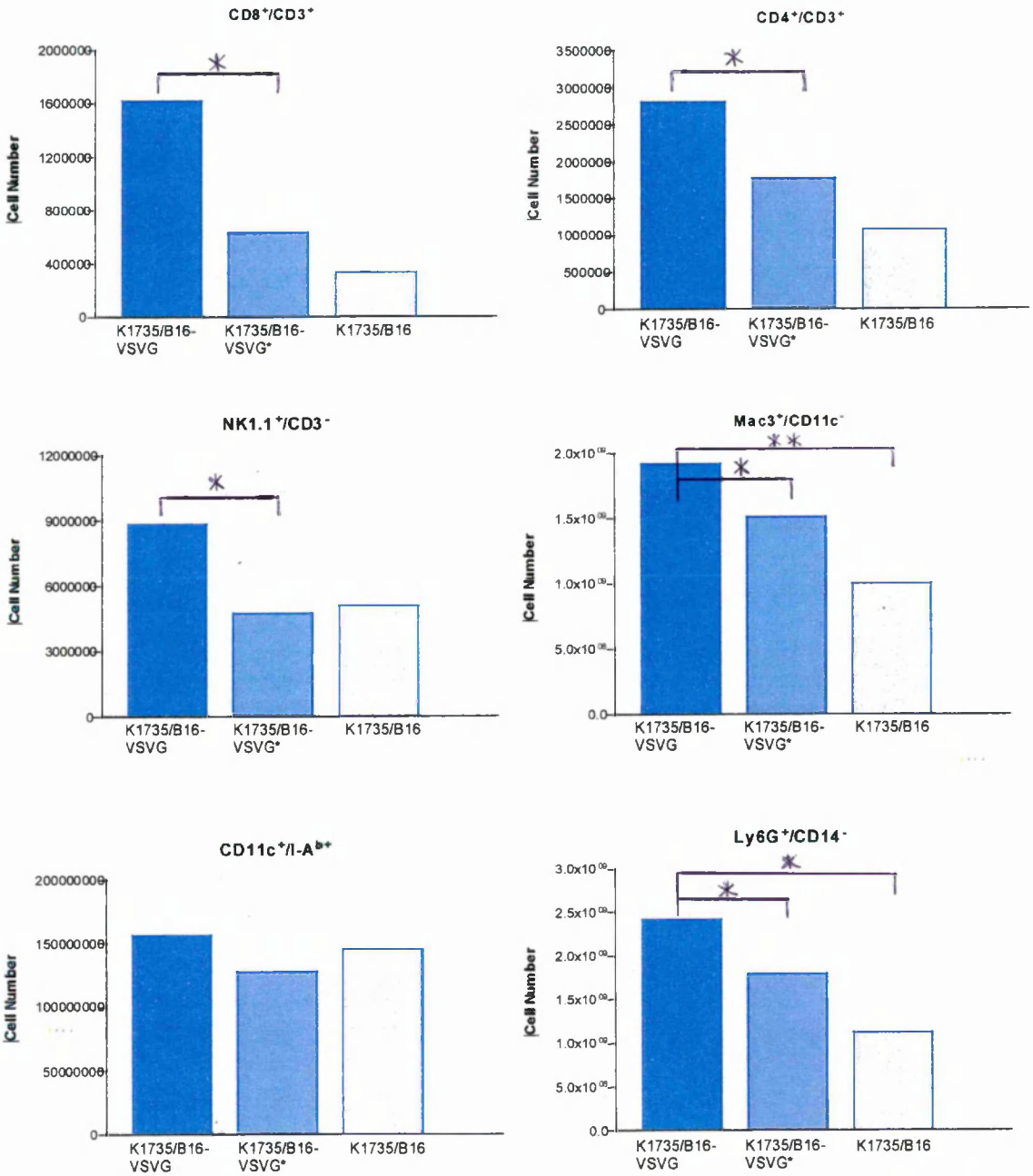


Figure 6.1: VSV-G-mediated fusion enhances infiltration of immune cells at the site of vaccination.

Irradiated K1735/B16-VSVG, K1735/B16-VSVG*, or K1735/B16 vaccine cells were injected into implanted sponges in mice, 48 hrs later the sponges were harvested and infiltrating cells were analysed as previously described (Chapter 4). The trends shown here (except for CD4+/CD3+) are representative of three individual experiments (*$P<0.09$, **$P<0.005$).

These data suggest that VSVG-induced fusion creates a more inflammatory local environment leading to enhanced recruitment of APC and effector cells, which may contribute to the enhanced *in vivo* efficacy of the fusing K1735/B16 vaccine compared to the non-fusing VSVG-expressing or the unmodified vaccines.

6.2 Allogeneic MHC Class I is a critical factor in the immunogenicity of syncytial cell killing.

Based on our observation that the presence of K1735 allogeneic cells as the fusion partner is necessary for vaccine potency in our fusing tumour cell vaccines (Chapter 5), we investigated the role of allogeneic MHC Class I molecules in the immunogenicity of a fusing vaccine. For these studies we utilized B16-derived cell lines expressing no detectable syngeneic MHC I on their surface (B16-neg), or engineered to express high levels of autologous H-$2D^b$ (B16-syng) or allogeneic H-$2L^d$ (B16-allo) (Figure 6.2) (Thomas, Greten et al. 1998). Fusing and non-fusing vaccines of each of these cell lines were prepared as previously described (Chapter 5 and materials and methods), irradiated with 100Gy and tested in a protection vaccination model in C57BL/6 mice. Mice were challenged with live B16-syng cells and animal survival post challenge was followed. Expression of VSVG without any fusion and a syngeneic MHC I molecule by B16 cells (B16syng-VSVG*) had no significant effect on the immunogenicity of the vaccine (Figure 6.3). Similarly, expression of VSVG without any fusion by B16 expressing no MHC I (B16neg-VSVG*) did not enhance the immunogenicity of such a vaccine. Expression of VSVG without any fusion in combination with allogeneic MHC I (B16allo-VSVG*) however, significantly enhanced the immunogenicity of the vaccine compared to either B16neg-VSVG* ($P<0.002$) or B16syng-VSVG* ($P<0.002$). Fusion of B16-neg to each other by VSV-G expression (B16neg-VSVG) generated a significant therapeutic benefit relative to B16neg-VSVG* ($P<0.002$) and fusion of B16-syng to each other by VSV-G led to an enhanced yet not statistically significant therapeutic effect relative to B16syng-VSVG*. But most importantly, the fusion of B16-allo cells to each other by VSV-G led to potent enhancement of the therapeutic effect, with 90% of mice surviving tumour challenge long term (>60 days) ($P=0.04$ relative to B16allo-VSVG*), comparable to protection observed with B16/K1735 fusing vaccine. Although the extent to which allogeneic MHC or VSVG-mediated fusion

contribute individually to the immunogenicity of a tumour cell vaccine needs further investigation, the data in figure 6.3 show that expression of an allogeneic MHC molecules on the surface of fusing tumour cells significantly enhances the efficacy of such a vaccine. This suggests that allogeneic MHC molecules expressed by K1735 in the fusing K1735/B16 vaccines contribute significantly in the immunogenicity of this vaccine.

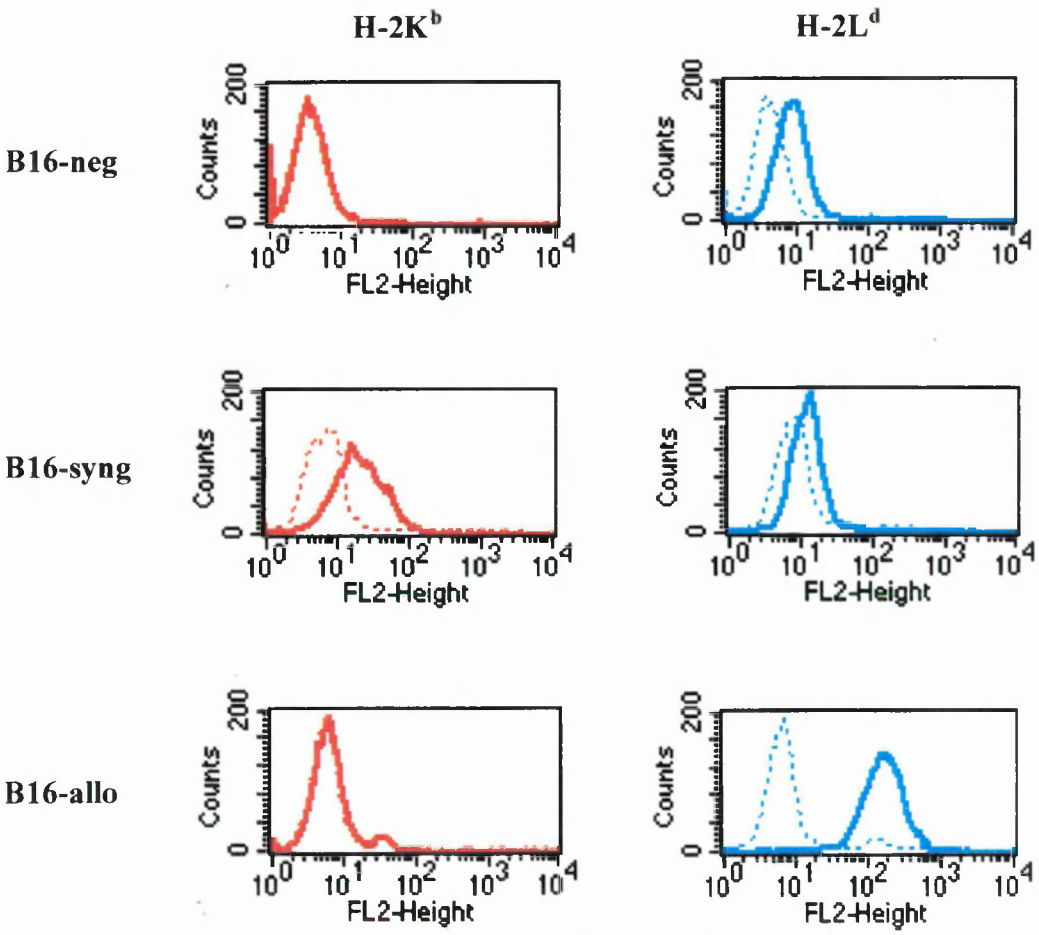


Figure 6.2: Profile of MHC Class I surface expression by B16 Class I variants. 1-5x10^6 B16-neg, B16-syng and B16-allo tumour cells were stained with antibodies against H-2K^b (solid red line) or H-2L^d (solid blue line) and analysed by multicolor FACS analysis. Appropriate control IgG staining shown as dashed lines.

p values	B16neg-VSVG	B16syng-VSVG	B16allo-VSVG	B16neg-VSVG*	B16syng-VSVG*	B16allo-VSVG*
B16neg-VSVG		NS		0.002	NS	NS
B16syng-VSVG			0.04	0.002	NS	NS
B16allo-VSVG				0.0001	0.001	0.04
B16neg-VSVG*					NS	0.002
B16syng-VSVG*						NS
B16allo-VSVG*						

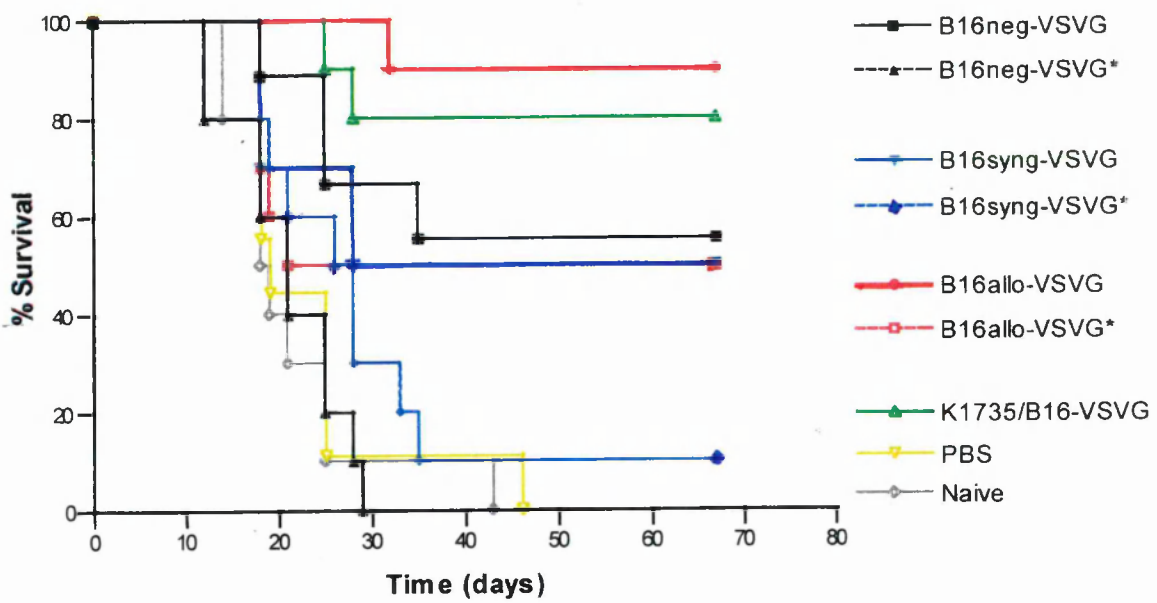


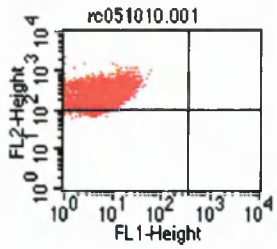
Figure 6.3: Allogeneic MHC Class I is a critical factor for the immunogenicity of syncytial cell killing.
Fusing (VSVG) and non-fusing (VSVG*) vaccines of B16-neg, B16-syng and B16-allo were prepared, irradiated with 100Gy and injected into groups of 10 C57BL/6 mice as previously described. Mice were then inoculated with live B16-syng cells and animal survival was monitored post tumour challenge. Control groups received PBS as a vaccine or no treatment. K1735/B16-VSVG vaccine was used as a comparison for efficient protection.

6.3 Syncytial cell killing enhances uptake of tumour derived material by both macrophages and immature dendritic cells.

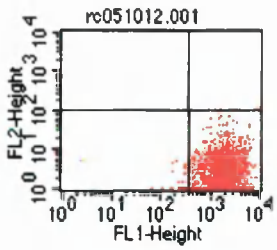
Previous data from our laboratory has demonstrated an important role for macrophages in coordinating immune responses to tumour vaccines (Gough, Melcher et al. 2001). In view of the data described in Chapter 4 and **Figure 6.1** where elevated macrophage numbers infiltrate the fusing K1735/B16 vaccine site, we hypothesised that these cells may play an important role in mediating the efficacy of this approach. Peritoneal macrophages were prepared as described in Materials and Methods. Co-culture of labelled macrophages (**Figure 6.4.Ai**) with labelled tumour cells (**Figure 6.4.Aii**) showed that macrophages can take up tumour derived material to a small degree from actively growing cultures (seen as a right shift of staining of the macrophages in **Figure 6.4.Aiii**). When autologous B16 tumour cells were killed through syncytial formation, uptake of labelled material by the macrophages was occasionally enhanced (**Figure 6.4.Av**). However, uptake of tumour derived material was consistently optimal when the target cells contained allogeneic cells (K1735 alone or K1735/B16), (**Figure 6.4.Aiv**). No secretion of either IFN-γ or IL-10 could be detected from macrophages co-cultured with syncytia. TNF-α was produced at low levels by macrophages in response to K1735/B16 mixtures in the absence of cell fusion (**Figure 6.5.Aiii**) or B16 cells in the presence of fusion (**Figure 6.4.Av**). However, TNF-α production was consistently seen at highest levels in response to fusing cells containing allogeneic cells (**Figure 6.4.Aiv**).

Similar experiments with immature murine dendritic cells showed enhanced levels of uptake of tumor material from fusing cells compared to normal cultured cells, but there was no distinction between fusing cell mixes which contained only autologous or allogeneic components (**Figure 6.4.B**). Incubation of immature DC with syncytia did not induce TNF, IL-10 or INF-γ secretion under any conditions tested.

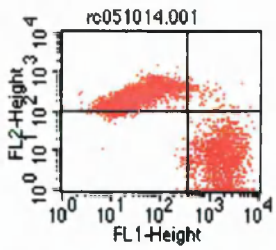
A.



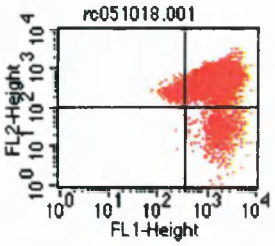
i) Macrophages, red



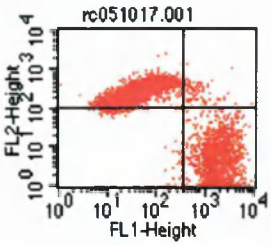
ii) B16/1735, green



iii) B16/1735 + Macrophages 10pg TNF

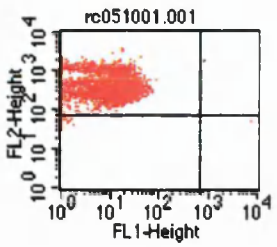


iv) K1735/B16-VSVG + Macrophages 76pg TNF

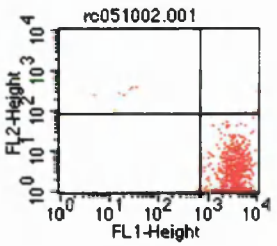


v) B16/B16-VSVG + Macrophages 5pg TNF

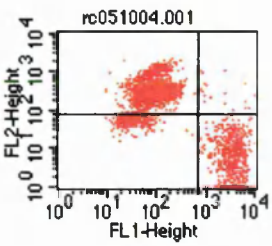
B.



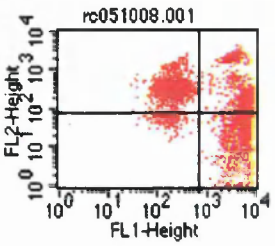
i) DC, red



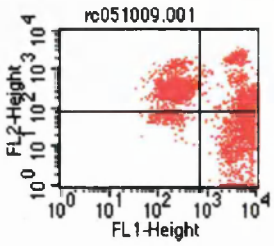
ii) B16/1735, green



iii) B16/1735 + DC



iv) K1735/B16-VSVG + DC



v) B16/B16-VSVG + DC

Figure 6.4: Syncytial killing enhances uptake of tumour derived material by both macrophages and IDC.

A. Red labeled peritoneal macrophages (i) or green labeled tumour cells (ii) were co-cultured (iii) or were co-cultured following transfection with VSV-G and acid shock to induce syncytial fusion and cell killing (iv and v). The resulting populations were analysed by FACS for populations of single or double labeled cells. The release of TNF-α was measured by ELISA and figures are shown per 10^5 fresh C57BL/6 peritoneal macrophages/ml/24 hrs. **B.** As for A except that tumour cells were co-cultured with IDC.

6.4 Syncytial cell killing leads to cross presentation by dendritic cells of a model tumour antigen from both autologous and allogeneic tumour cells.

We investigated further the immunological mechanisms by which syncytial killing leads to the anti tumour immunity that we have observed in these studies. T cells from OT1 transgenic mice recognise the SIINFEKL epitope of the ovalbumin antigen in the context of H-2^b (Hogquist, Jameson et al. 1994). DC loaded with SIINFEKL peptide are recognised by OT1 cells leading to INF-γ release (**Figure 6.5.A**). Due to low levels of MHC Class I expression B16ova-transfected cells cannot present sufficient SIINFEKL epitope to OT1 cells from endogenous expression of ova (**Figure 6.5.A**) (unless pre-treated with INF-γ to upregulate Class I MHC expression, data not shown) for detectable cytokine release. Similarly, B16-ova incubated with DC in the absence of killing do not release sufficient ova epitopes for presentation (**Figure 6.5.A**). Lysates of B16ova cells killed by freeze thawing or osmotic shock were also unable to load DC with the appropriate epitope of ova for cross presentation to OT1 cells (data not shown). In one of three experiments, B16ova cells killed by irradiation (100Gy) were able to serve as a source of ova antigen for DC in a cross presentation assay (**Figure 6.5.B**). However, B16ova cells fused by VSV-G were a reproducible and potent source by which the ova epitope could be supplied to DC for subsequent presentation to OT1 cells (**Figure 6.5.B**). This presentation did not occur in the absence of DC to cross-present the antigen. This demonstrates that fusion of tumour cells leads enhanced cross presentation of tumour derived antigens by DC and thus enhanced crosspriming of T cells.

Despite repeated attempts using both plasmid and retroviral-mediated gene transfer of the ova gene, we were unable to generate a K1735 cell line expressing ova. We were, however, able to generate NIH3T3 cells expressing ova which could serve as an allogeneic source of the protein (NIH3T3 cells are unable to present ova to OT1 cells even with INF-γ stimulation due to a lack of H2b MHC). To a lesser degree than with fusing B16ova cells, NIH3T3ova cells expressing fusogenic VSV-G loaded H-2^b+ve DC with ova which led to presentation of the SIINFEKL epitope to OT1 cells (**Figure 6.5.C**). In these experiments, irradiation (**Figure 6.5.C**), freeze thaw or osmotic shock (data not shown) of the allogeneic ova expressing line, were ineffective at promoting cross presentation of the ova antigen by $H2^b$ DC.

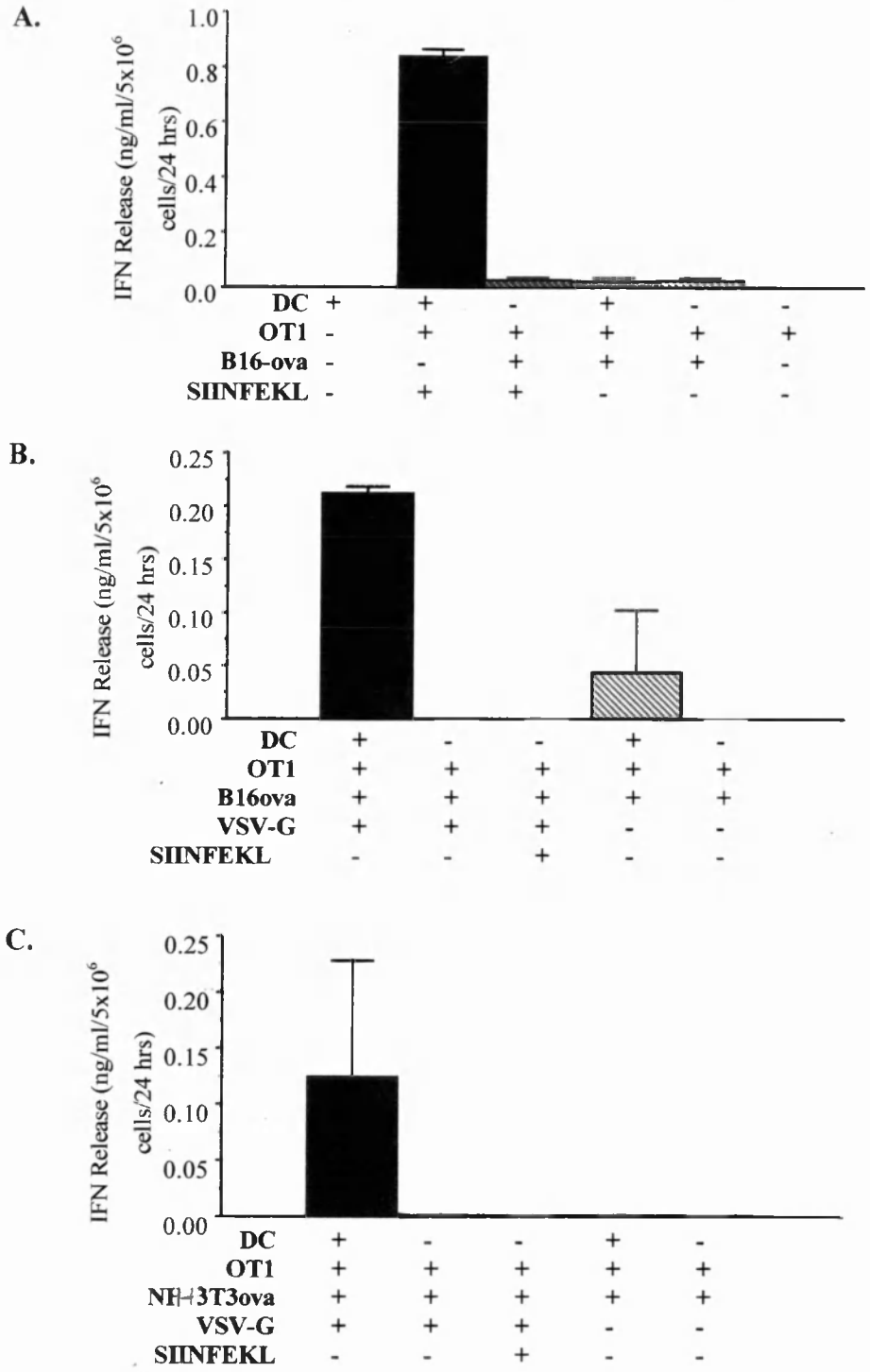


Figure 6.5: Syncytial cell killing leads to cross presentation by dendritic cells of a model tumour antigen from both autologous and allogeneic tumour cells .
A. OT1 T cells were co-cultured for 24 hours with various mixes of tumour and dendritic cells as shown. As controls, OT1 cells were co-cultured with immature C57BL/6 dendritic cells (DC) either alone or pre-loaded with the SIINFEKL peptide as a positive control for recognition; or with B16ova tumour cells or with mixtures as indicated. IFN-γ release from the OT1 cells was measured by ELISA as shown. **B.** As for **A** except that B16ova were VSV-G transfected and fused in some samples. **C.** As for **B** except that the tumour cells in this case were NIH3T3ova allogeneic cells.

6.5 Summary

Irradiated VSV-G fusing tumour cell vaccines attract NK, $CD8^+$ T cells, macrophages, neutrophils and possibly $CD4^+$ T cells, compared to VSV-G expressing, but non-fusing, vaccines or just irradiated vaccines. This observation further supports the suggestion that the mechanism of syncytial death is more immunostimulatory than irradiation alone despite the presence of the potential viral immunogen. More evidence to support this comes from our observations that syncytial killing enhances the uptake of tumour derived material by macrophages and dendritic cells and also leads to efficient cross-priming of T cells by DC *in vitro*.

Chapter 7

Transcriptional Control of FMG Expression for the Development of Stable Cell Lines Expressing FMGs.

We have shown that FMGs are able to potently enhance the immunogenicity of a combined autologous and allogeneic cell vaccine. To develop this for therapeutic application we aimed to develop allogeneic cells stably expressing FMGs as an "off the shelf" reagent either to combine with patient derived tumour material or to inject directly within established tumour nodules. As previously demonstrated however, expression of a FMG gene under the control of a strong constitutive promoter, such as CMV, in cells expressing the appropriate FMG-receptor leads to potent direct and bystander cytotoxic effects (Chapter 5, (Bateman, Bullough et al. 2000; Diaz, Bateman et al. 2000; Fielding, Chapel-Fernandes et al. 2000)). Therefore, for the purposes of generating stable FMG-expressing cells lines it is essential to regulate gene expression

The "Tet-On" transcription regulation system (Gossen, Freundlieb et al. 1995; Baron, Gossen et al. 1997; Rossi and Blau 1998) (as described in Introduction) was utilised in these studies to regulate expression of FMGs. Briefly, the system is composed of two critical components and gene expression is regulated by the tetracycline analogue Dox (**Figure 7.1**). The first component is the reverse tetracycline-controlled transcativator (rtTA), a hybrid transcription factor that can specifically bind to the second key component, the TRE. The TRE contains sequences from the tetracycline operator and is coupled to a minimal CMV promoter, forming a tet-responsive promoter. A FMG gene is placed under the control of this promoter but in the absence of Dox, the rtTA is unable to bind to the TRE and activate transcription. Addition of Dox leads to conformational changes in the rtTA which enable it to bind to the TRE and consequently induce transcription.

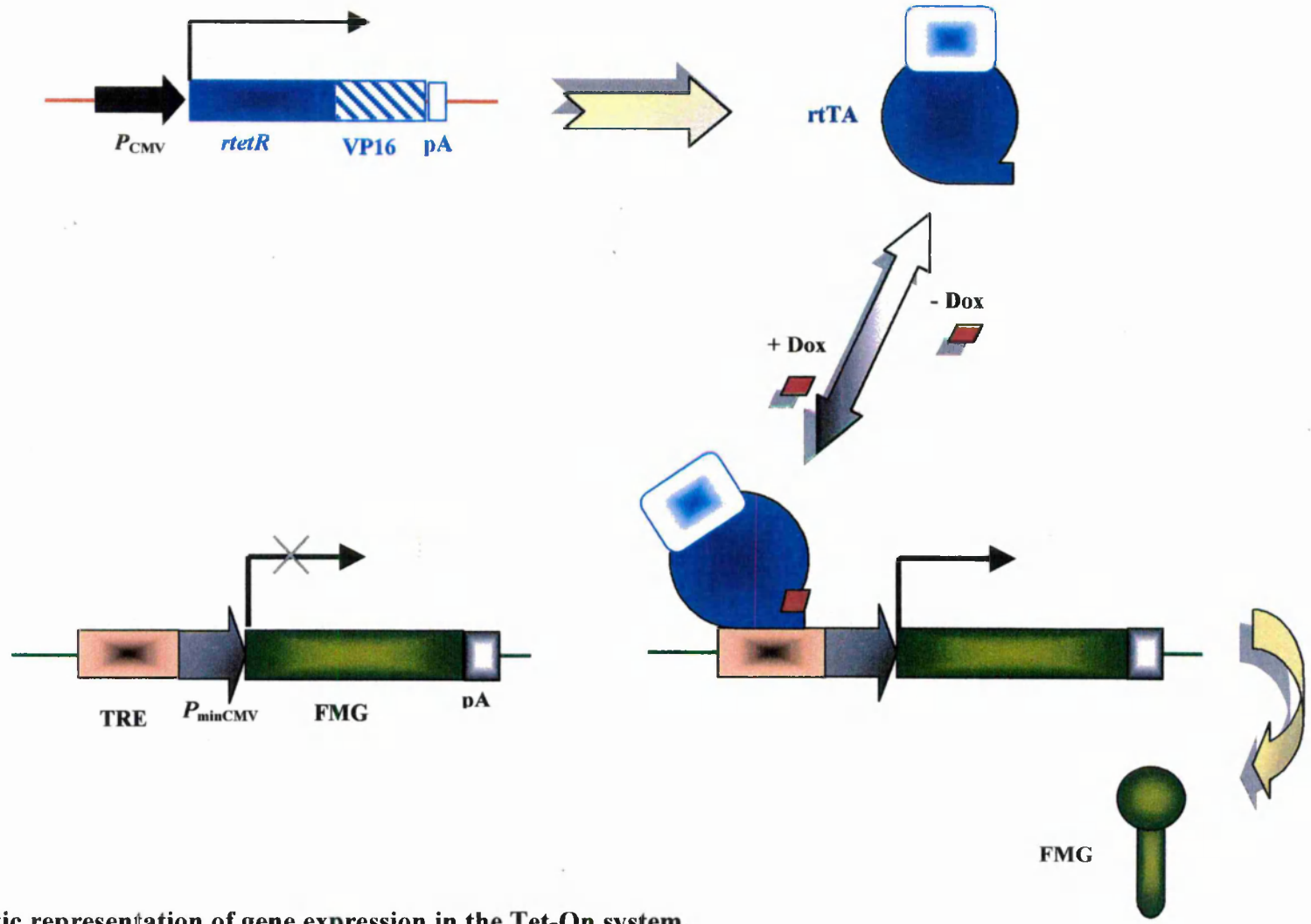


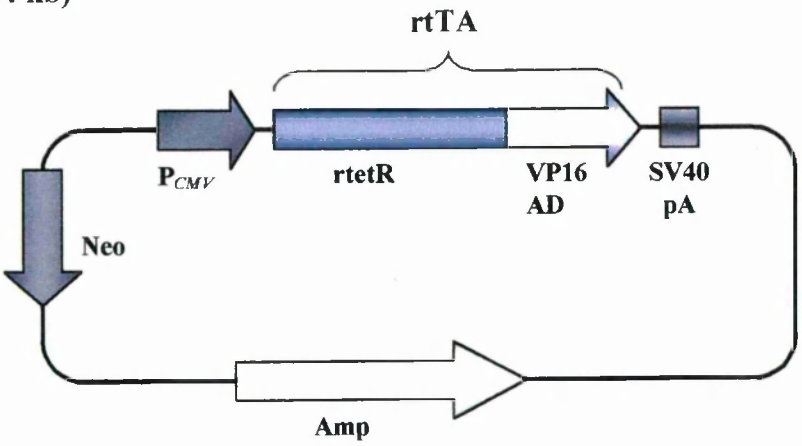
Figure 7.1: Diagrammatic representation of gene expression in the Tet-On system.
Binding of Dox to the reverse tetracycline transcriptional activator (rtTA) induces a conformational change in the rtTA molecule that allows it to bind the TRE and activate transcription

7.1 Cloning of the hyperfusogenic GALV envelope gene in the Tet-On system regulation plasmids.

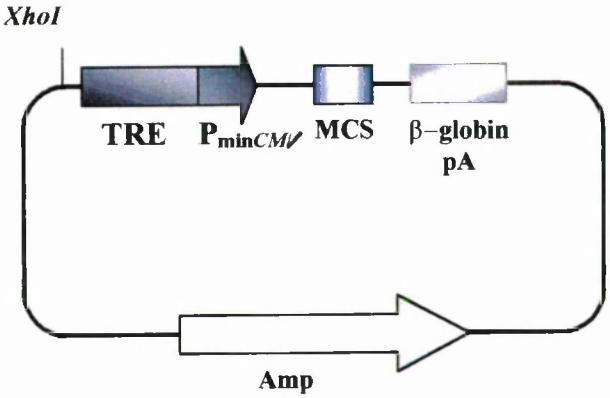
The Tet-On system (Clontech) was used to regulate expression of the GALV FMG in a panel of different human cell lines. The GALV envelope gene used in these studies expresses a hyperfusogenic C-terminally truncated mutant of the wild type envelop protein (Fielding, Chapel-Fernandes et al. 2000). Expression of GALV on human cells expressing the receptor for GALV, PitI, leads to extensive syncytia formation and potent direct and bystander cytotoxic effects within 24 hrs. The key plasmids used in these studies are shown in **Figure 7.2A**. pTet-On plasmid (Gossen, Freundlieb et al. 1995) expresses the rtTA regulator protein from the strong immediately early promoter of CMV. In addition, this plasmid contains a neomycin resistance gene for selection of transduced cells. The pTRE response plasmid (Resnitzky, Gossen et al. 1994) contains the TRE coupled to the minimal CMV promoter (**Figure 7.2B**). Immediately downstream of this Tet-responsive promoter is a multiple cloning site (MCS) consisting of unique restriction enzyme sites for cloning of the gene of interest in the plasmid. Generation of cell lines expressing GALV under the control of Tet involves the development of double-stable cell populations transduced initially with the pTet-On regulator plasmid for stable expression of the rtTA, followed by transduction with the pTRE response plasmid carrying the GALV gene for Tet-regulated expression of GALV.

For the purposes of cloning of the GALV envelope gene into the pTRE plasmid, *BamHI* sites were created on either end of the GALV gene using PCR and appropriately designed primers (see Materials and Methods). The 2.2 Kb PCR product corresponding to the GALV gene containing *BamHI* sites on either side was inserted into the shuttle plasmid pCR3.1 (Invitrogen) to create plasmid pCR3.1-GALV. The GALV gene was subsequently excised from pCR3.1-GALV by *BamHI* digestion and ligated to a *BamHI*-linearised pTRE plasmid, to yield plasmid pTRE-GALV (Figure 7.2C). Sequencing of the GALV gene contained in pTRE-GALV confirmed the correct orientation of the GALV, as well as the integrity of the gene in the final plasmid.

A. pTet-On (7.4 kb)



B. pTRE (3.1 kb)



C. pTRE-GALV (5.3 kb)

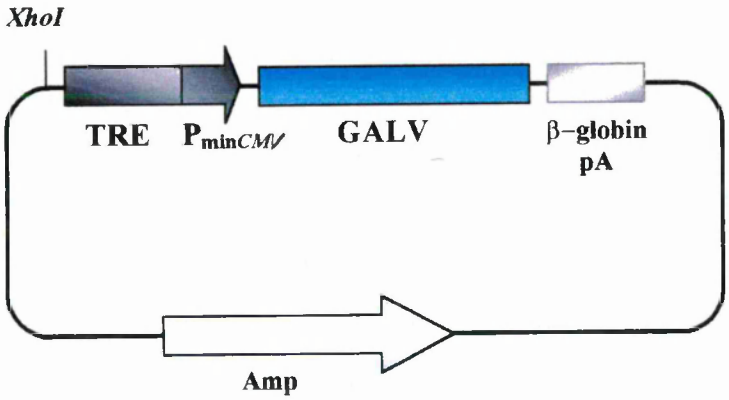


Figure 7.2: Maps of plasmids used in the Tet-On system.
A: pTet-On regulator plasmid. **B:** original pTRE response plasmid. **C:** modified pTRE-GALV response plasmid. P_{CMV} immediate early cytomegalovirus promoter; rtetR: reverse tetracycline repressor; VP16 AD: activation domain of VP16 protein of herpes simplex virus; pA: poly A signal; Amp: ampicilin resistance gene; Neo: neomycin resistance gene; TRE: tetracycline response element; P_{minCMV}: minimal CMV promoter; MCS: multiple cloning site.

7.2 Regulation of GALV expression in 293 cells.

To demonstrate the proof-of-principle of using the Tet-On system for regulating gene expression in human cells, a modified human primary kidney embryonal cell line, 293-On, was initially used in the studies. The 293-On cell line (Clontech) is a subclone of 293 cells stably transformed with the pTet-On regulator plasmid and expressing high levels of rtTA. To verify the inducibility potential of the 293-On cells, the cells were transfected with a pTRE plasmid expressing the reporter gene for luciferase, pTRE-Luc (Clontech), using the Effectene transfection system as described in Materials and Methods. For Tet-induced gene expression, 1μg/ml of Dox was provided in the media of the transfected cells immediately following transfection and luciferase expression was monitored over 72 hrs by a luciferase assay. To determine the basal level of gene expression from the pTRE plasmid in this cell line, the cells were transfected with pTRE-Luc in the absence of Dox. No transfection or transfection with "empty" pTRE plasmid were used as controls. **Figure 7.3** shows that in the presence of Dox luciferase expression can indeed be induced to high levels compared to the negative controls. However, relatively high levels of basal luciferase expression were observed in the absence of Dox and overall luciferace expression upon Dox administration could only be induced 3-4 fold higher than the background expression. These data demonstrate that although Dox can significantly induce gene expression in the 293-On cell line, gene expression is not completely silent in the absence of Dox.

Since the relevance of basal gene expression is a function of the gene selected, we next proceeded to test the pTRE-GALV and determine the basal level of GALV expression plasmid in the same transient transfection system used above. 293-On cells were transfected with pTRE-GALV and the level of GALV expression was determined by level of syncytia formation. pCR3.1-GALV was used as a positive control for GALV fusion. The levels of syncytia formation were determined by visual observation under the microscope (**Figure 7.4**) and are summarised in **Table 7.1**. Transfection of 293-On cells with pCR3.1-GALV led to rapidly progressive fusion (**Figure 7.4A**). Approximately 60% of the cells were fusing at 12 hrs, and by 24 hrs almost 100% fusion could be observed. However, syncytia formation was also

observed in the cells transfected with the pTRE-GALV in the absence of any Dox (**Figure 7.4B**). Although the extent of fusion was limited at the initial timepoints (approximately 15% at 24 hrs), it significantly increased at the later timepoints of the experiment and by 96 hrs approximately 80% of the cells had formed syncytia. No syncytia formation was observed in the mock transfected cells (**Figure 7.4C**). The results of this experiment confirm that the GALV gene cloned in the pTRE-GALV plasmid is functional, but, most importantly, they demonstrate the existence of significantly high basal levels of GALV expression in the absence of Dox from the Tet-responsive plasmid at the level of transient expression in 293-On cells. This apparent "leakiness" of the Tet-On system in regulating GALV expression following transient transfection may present a significant hurdle in the development of cellular or viral vectors expressing GALV or other FMG genes.

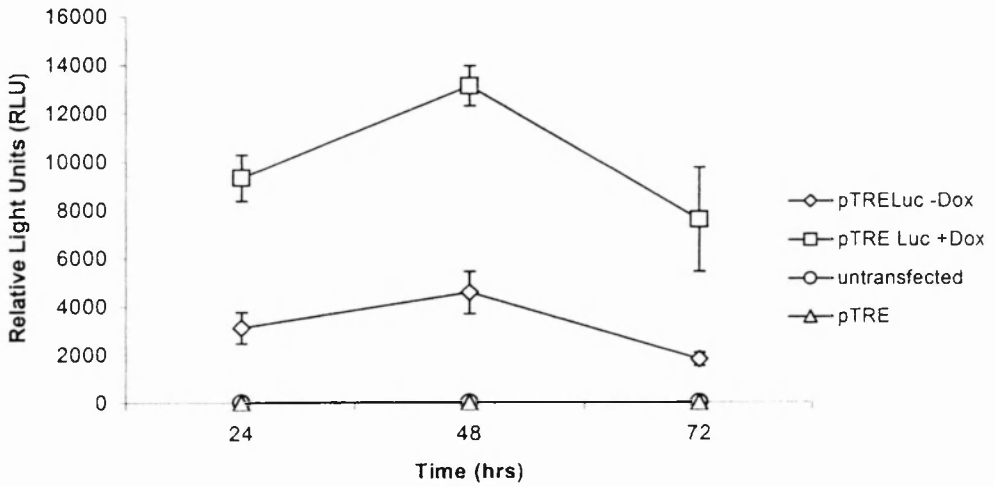


Figure 7.3: Luciferace expression in 293-On cells transiently transfected with pTRE-Luc.
293-On cells were transfected with plasmid pTRE-Luc in the presence or absence of 1 μg/ml of Dox, plasmid pTRE or nothing. 24, 48 and 72 hrs post transfection protein was isolated from cells and analysed for Luciferase activity.

	Timepoints (hrs)				
Samples	**12**	**24**	**48**	**72**	**96**
pCR3.1-Galv	+++	+++++	+++++	+++++	+++++
pTRE-Galv	-	+	++	+++	++++
untransfected	-	-	-	-	-
no DNA	-	-	-	-	-

Table 7.1: Level of fusion of 293-On cells transiently transfected with pTRE-GALV.
293-On cells were transfected with the indicated plasmids and the extent of syncytia formation was monitored over 72 hrs. The extent of syncytia formation was used as a measure of GALV gene expression levels. -: 0% fusion; +: 1-20%; ++: 21-40%; +++: 41-60%; ++++: 61-80%; +++++: 81-100%.

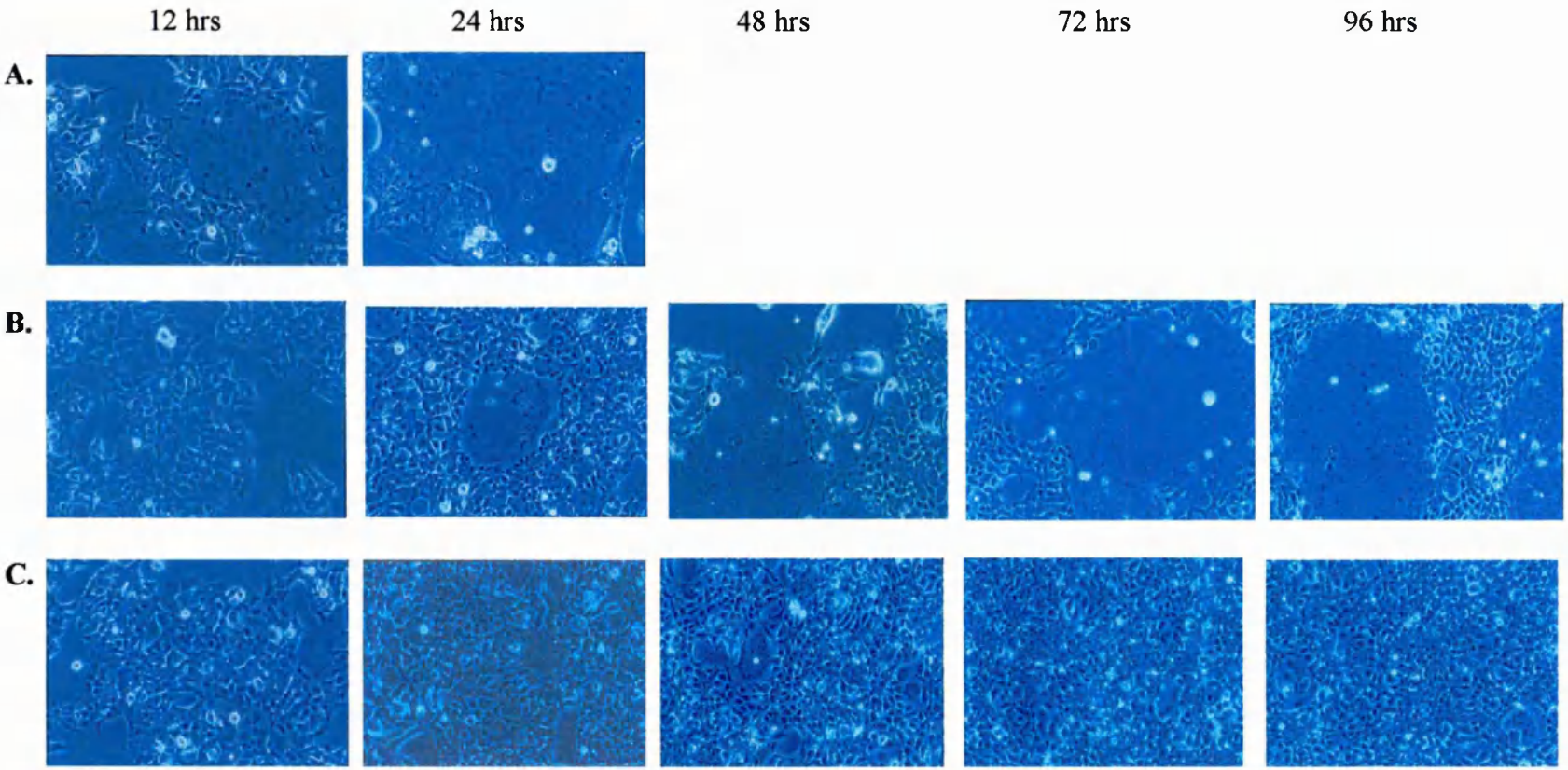


Figure 7.4: Syncytia formation in 293-On cells transiently transfected with pTRE-GALV.
293-On cells were transfected with plasmid **A.** pCR3.1-GALV, **B.** pTRE -GALV, or **C.** mock transfected. Syncytia formation was monitored at 12, 24, 48, 72 and 96 hrs post transfection using phase contrast light microscopy. Representative pictures show extent of syncytia formation in each sample. Note: No pictures available for panel A 48, 72 and 96 hrs as cells had fused completely and detached from plates.

7.3 Generation of double-transfected 293-On cell lines expressing GALV.

Since our goal is the generation of long-lived stable cell lines conditionally expressing GALV, we decided to investigate if it was possible to isolate clonal populations of 293-On cells transformed with pTRE-GALV that exhibit low background, yet high induction potential of GALV expression. Plasmid pTRE expresses no drug selectable marker for selection of stable drug-resistant populations. 293-On cells were therefore co-transfected with pTRE-GALV and pBabePuro, the latter plasmid conferring puromycin resistance, at a ratio of 20:1 respectively and 48 hrs following transfection the cells were subjected to puromycin selection (1μg/ml Puro). The use of 20 times more response plasmid compared to selection plasmid significantly enhances the likelihood that cells resistant to puromycin have also integrated a copy or copies of the response plasmid. Approximately 2 to 3 weeks later puromycin-resistant transformed colonies could be isolated and further expanded in individual cultures. A total of 48 sub-clones were analysed for induction of GALV expression and syncytia formation in the presence or absence of Dox. Cells were plated in duplicates and the first set of cells was kept in normal tissue culture media while the second set was kept in 1μg/ml of Dox. Light microscopy was used to monitor extent of fusion over 96 hrs and results from 22 representative clones are summarised in **Table 7.2**. 10 of the 22 sub-clones exhibited no background and no induction of GALV expression as demonstrated by the lack of syncytia formation in the absence or presence of Dox. Six of the sub-clones exhibited very low background and low levels of induced GALV expression. Two of the sub-clones exhibited medium to high levels of GALV induction, but the levels of background expression were also high, while two subclones exhibited low background expression and medium induced expression. Finally, two of the sub-clones exhibited very low levels of background GALV expression with high induction potential. In the absence of Dox less than 5% of cells of sub-clones 26 and 28 were involved in syncytia at any timepoint. By 72 hrs of Dox stimulation approximately 90% of cells of sub-clone 26 were forming syncytia, while by the same timepoint approximately 80% of the cells of clone 28 were forming syncytia. These results demonstrate that upon continuing puromycin selection clones of 293-On cells with minimal background levels of GALV expression and high induction potential can be developed.

	- Dox				+ Dox			
Sub-clone	24 hrs	48 hrs	72 hrs	96 hrs	24 hrs	48 hrs	72 hrs	96 hrs
1	-	-	-	-	-	-	-	-
7	+	+	+	+	+	+++	+++	+++
8	++	++	++	++	++	+++	++++	++++
9	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-
16	+	+	+	+	+	++	++	++
17	+	+	+	+	+	+	+	+
19	-	-	-	-	-	+	+	+
20	++	++	++	++	++	++	++	+++
22	-	-	-	-	+	+	+	+
23	-	-	-	-	-	-	-	-
24	-	-	-	-	-	-	-	-
26	+	+	+	+	+	++	+++	+++++
27	-	-	-	-	-	-	-	-
28	-	+	+	+	-	++	+++	+++++
40	+	+	+	+	+	+	+	+
41	-	+	+	+	-	+	+	+
42	-	-	-	-	-	-	-	-
45	-	-	-	-	-	-	-	-
46	-	+	+	+	-	++	++	+++
47	-	-	-	-	-	-	-	-
48	-	-	-	-	-	-	-	-

Table 7.2: Level of syncytia formation in double-transformed 293-On sub-clones expressing GALV.
To determine the levels of induction of GALV expression in each individual 293-On GALV-expressing sub-clone, 1 μg/ml of Dox was administered to the cells and fusion was monitored by phase contrast light microscopy. Basal levels of GALV expression was determined by monitoring fusion in the absence of Dox. -: 0% fusion; +: 1-20%; ++: 21-40%; +++: 41-60%; ++++: 61-80%; +++++: 81-100%.

Gene expression from the Tet-response plasmid is dependent on the concentration of Dox. Although 1μg/ml of Dox is well documented in the literature as being an optimal dose for inducing gene expression in various models, we determined whether the lack of fusion induction observed in some of our clones was due to sub-optimal levels of Dox. Clones 19, 23, 26 and 28 were plated in increasing amounts of Dox and the extent of fusion was assessed 72 and 96 hrs later (**Table 7.3**). 100 μg/ml of Dox was toxic to all the cells and cells died within 24 hrs of exposure to this concentration. Although 10 μg/ml of Dox was not potently cytotoxic a reduction in the growth rate of the cells was observed. The remaining Dox concentrations had no

apparent direct toxic effect on the cells. 10 μg/ml of Dox did not further enhance the extent of fusion compared to 1 μg/ml of Dox in any of the clonal cell lines. Induction of fusion was therefore determined to be optimal at 1 μg/ml of Dox and lack of fusion in the clones tested was not due to sub-optimal Dox levels.

72 hrs	Dox concentrarion (μg/ml)					
clone	0	0.01	0.1	1	10	100
1	-	-	-	-	-	cells dead
9	-	-	-	-	-	cells dead
23	-	-	-	-	-	cells dead
7	+	+	+	+++	+++	cells dead
26	+	+	+	+++	+++	cells dead
28	+	+	++	+++	+++	cells dead

96 hrs	Dox concentrarion (μg/ml)					
clone	0	0.01	0.1	1	10	100
1	-	-	-	-	-	cells dead
9	-	-	-	-	-	cells dead
23	-	-	-	-	-	cells dead
7	+	+	+	+++	++++	cells dead
26	+	+	++	+++++	+++++	cells dead
28	+	+	+++	++++	+++++	cells dead

Table 7.3: Effect of Dox concentration on levels of fusion in 293-On cell lines transformed with pTRE-GALV.
293-On sub-clones were plated in 0, 0.01, 0.1, 1, 10 and 100 μg/ml of Dox. 72 and 96 hrs later extent of fusion was determined by light microscopy. -: 0% fusion; +: 1-20%; ++: 21-40%; +++: 41-60%; ++++: 61-80%; +++++: 81-100%.

7.4 Regulation of GALV expression in Hela cells.

The specificity and efficiency of the Tet-dependent regulation systems are known to be cell line specific (Gossen and Bujard 1995), with background levels of gene expression and maximal induction levels varying widely between different cell lines. Concurrent to our studies in the 293-On cells, the efficiency of the Tet-On system was investigated in an additional human cell line. Hela-On cells are a sub-clone of a

human cervical carcinoma cell line, transformed to express high levels of rTA. To determine the background levels of gene expression obtained in this cell line, Hela-On cells were transfected with pTRE-GALV and the extent of syncytia formation was determined at 48 hrs post transfection (**Figure 7.5**). A high level of background fusion was also observed in the bulk transfected Hela-On cells in the absence of Dox. Extent of fusion reached 50-60% by 48 hrs compared to zero fusion in the un-transfected control.

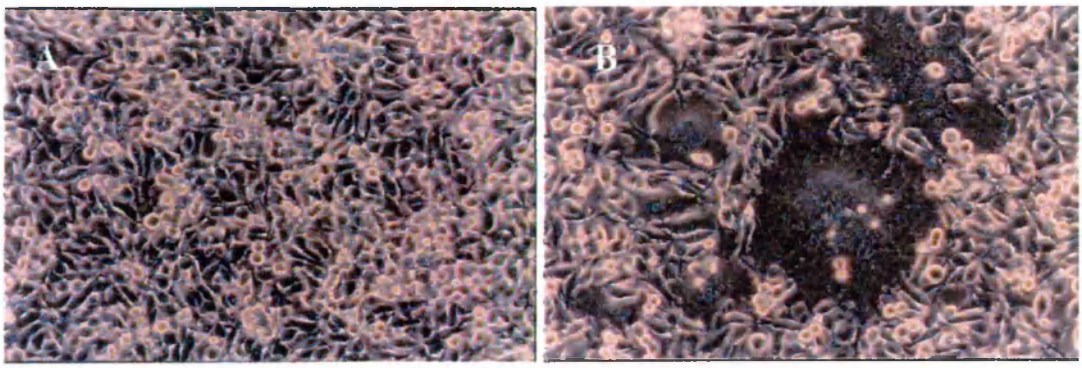


Figure 7.5: Syncytia formation in Hela-On cells transiently transfected with pTRE-GALV.
Phase contrast microscopy of **A.** parental, unmodified Hela-On cells or **B.** pTRE-GALV transfected Hela-On cells 48 hrs post transfection.

The feasibility of generating double-stable transformed cell lines expressing GALV under the control of Dox was also investigated using this cell line. For these studies the pTRE-GALV vector was modified to carry the Puro resistance gene yielding plasmid pTRE-GALV-Puro. This modification served to enhance the efficiency of the tranformation of the target cells and increase the number of resulting Puro resistant clones. Hela-On cells were transfected with pTRE-GALV-Puro and 48 hrs later they were selected with 0.5 μg/ml Puro. 2-3 weeks later individual Puro-resistant colonies were isolated and further expanded. As previously described for the 293-On clones, the Hela-On clones were screened for GALV expression in a fusion induction assay by plating each clone in duplicate samples and adding 1 μg/ml Dox to one set of the cells. Fusion induction was monitored over 96 hrs and the results of representative clones are summarised in **Table 7.4**. Clones 1, 6 and 21 exhibited no background fusion yet significantly high levels of fusion upon Dox administration (**Table 7.4** and **Figure 7.6**) and were regarded as "highly positive" clones. Clone 6

responded the fastest to Dox stimulation compared to the other 2 clones as shown by the high levels of fusion observed by 24 hrs. Clone 9 exhibited no background fusion in the absence of Dox and low fusion induction in the presence of Dox and was regarded as a "weakly positive" clone. The remaining clones exhibited minimal to no fusion induction upon Dox stimulation at any of the timepoints tested.

Sub-	- Dox				+ Dox			
Clone	24 hrs	48 hrs	72 hrs	96 hrs	24 hrs	48 hrs	72 hrs	96 hrs
1	-	-	-	-	++	+++	+++++	+++++
2	-	-	-	-	+	+	+	+
3	-	-	-	-	-	-	-	-
6	-	-	-	-	++++	++++	++++	+++
9	-	-	-	-	+ +	+ +	+ +	+ +
10	-	-	-	-	-	-	-	-
13	-	-	-	-	+	+	+	+
16	-	-	-	-	-	-	-	-
21	-	-	-	-	+	+++	++++	+++++

Table 7.4: Level of fusion of double-stable Hela-On sub-clones expressing GALV.
To determine the levels of induction of GALV expression in each individual Hela-On GALV-expressing sub-clone cells were cultured in 1 μg/ml of Dox and syncytia formation was monitored by phase contrast light microscopy. Basal levels of GALV expression were determined by monitoring fusion in the absence of Dox. -: 0% fusion; +: 1-20%; ++: 21-40%; +++: 41-60%; ++++: 61-80%; +++++: 81-100%.

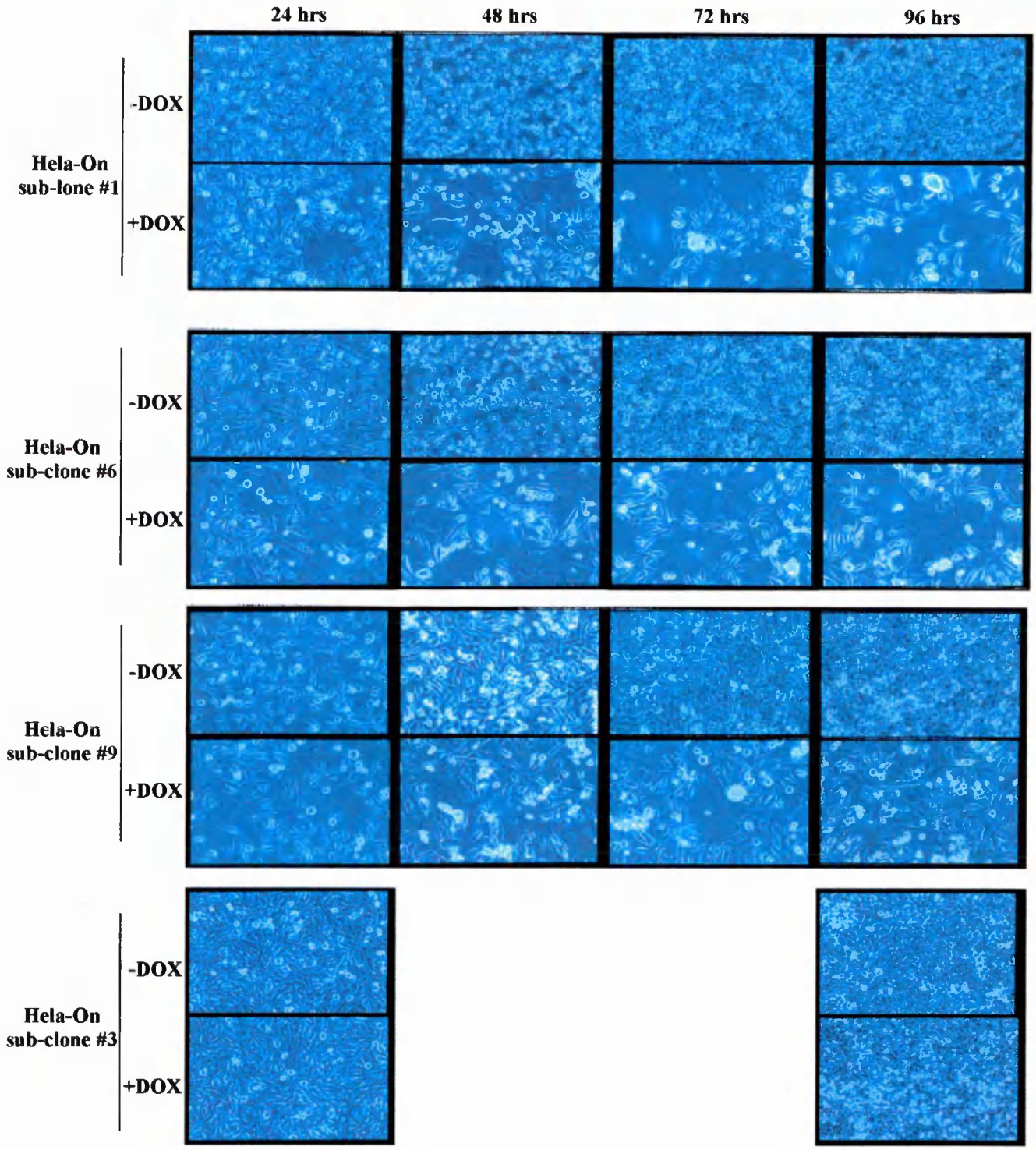


Figure 7.6: Syncytia formation in Hela-On double-stable sub-clones following Dox administration.

Hela-On sub-clones 1, 3, 6 and 9 were cultured in 1 μg/ml Dox or in Dox-free media and fusion was monitored over 96 hrs by light microscopy. Representative pictures are shown.

GALV expression levels in the presence or absence of Dox in the Hela-On-TRE-GALV clones were confirmed by a Northern Blot. Cells from selected clones (the negative clone 3, the weakly positive clone 9 and the highly positive clones 1 and 6) were cultured in media containing 1 μg/ml Dox or left untreated and RNA was extracted at 24, 48, 72 and 96 hrs following onset of Dox treatment and analysed by northern blot using a cDNA probe specific for GALV. No GALV expression could be detected in any of the clones in the absence of Dox treatment at any timepoint (**Figure 7.7**). As expected, in the presence of Dox no expression could be detected by clone 3, while high levels of GALV expression could be detected in clones 1, 6 and 9 at all timepoints tested. These results confirm the absence of background gene expression in the absence of Dox in these clones, as well as the high levels of inducibility in clones 1, 6 and 9.

The difference in the fusion levels observed among the transformed clones obtained after single cell clonal expansion may reflect a difference in the number of integrated copies of the pTRE-GALV-Puro plasmid from which gene expression could be induced. To determine the number of integrated copies of the GALV gene, genomic DNA was isolated from clones 1, 3, 6 and 9 and treated with the restriction endonuclease *XhoI*. This enzyme will cut the pTRE-GALV-Puro plasmid immediately upstream of the TRE (**Figure 7.2C**). The DNA was then analysed by Southern Blot using a cDNA probe specific for GALV. As expected, the parental non-transformed Hela-On cells did not carry any copy of the pTRE-GALV-Puro plasmid (**Figure 7.8**). Cells from clones 1, 6 and 9 all carried one copy of the response plasmid based on the single banding obtained. Interestingly, clone 3, which exhibited no fusion upon Dox stimulation, also carried one copy of the response plasmid. The difference in the expression levels and induction of GALV-mediated fusion among the clones analysed would therefore more likely be explained by differences in the site of integration of the plasmid: integration near highly active chromatin may result in higher gene expression levels whereas integration near a silent site may result in lower to absent gene expression. Alternatively, the integrated copy of TRE-GALV in clone 3 is not intact.

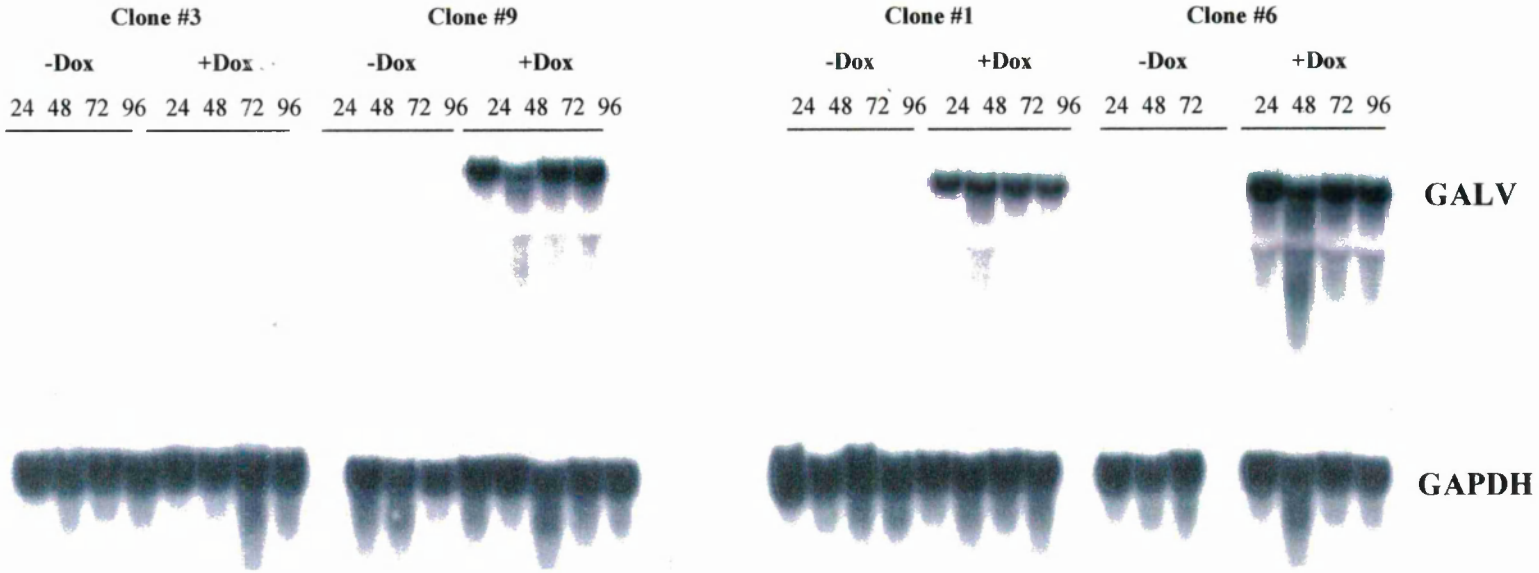


Figure 7.7: Northern blot analysis of GALV expression in Hela-On subclones.
Hela-On subclones 1, 3, 6 and 9 were cultured in media containing 0 μg/ml Dox (- Dox) or 1 μg/ml Dox (+ Dox) for 96 hrs. At 24, 48, 72 and 96 hrs of culture RNA was isolated from cells and analysed by Northern Blot using a cDNA probe specific for GALV. A GAPDH-specific probe was used to confirm equal loading of the samples.

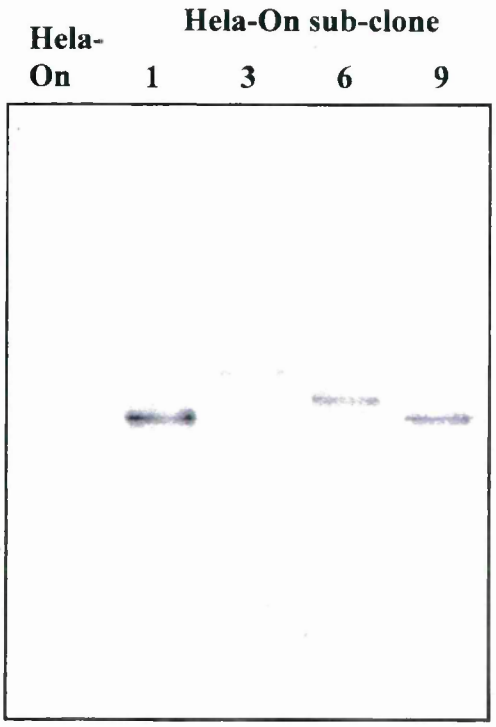


Figure 7.8: Southern Blot analysis of Hela-On sub-clones.
Genomic DNA from parental Hela-On cells and sub-clones 1, 3, 6 and 9 was isolated, treated with *XhoI* endonuclease and analysed by Southern Blot using a cDNA probe specific for GALV.

7.5 Reduction of basal levels of gene expression using a transcriptional silencer.

Basal levels of gene expression in the Tet-On system can be reduced by incorporation of a third component in the system, the tetracycline transcriptional silencer (tTS) (Freundlieb, Schirra-Muller et al. 1999). tTS can bind to the TRE in the response plasmid in the absence of Dox and actively inhibit initiation of transcription from the Tet-responsive promoter. As Dox is added in the cells, it binds tTS and induces a conformational change such that the silencer is no longer able to bind the TRE. At optimal Dox levels, the rtTA will be converted to its active form, associate with the TRE and stimulate transcription. 293-On cells were transiently transfected with pTRE-Luc alone or pTRE-Luc plus ptTS, a plasmid expressing the tTS, in the presence or absence of Dox and luciferase expression was monitored for a total of 72 hrs following transfection. As shown in **Figure 7.9** the basal levels of luciferase expression in the absence of Dox were reduced in the presence of tTS. Moreover, levels of gene expression upon Dox activation were higher in the presence of the tTS, resulting in significantly higher fold induction compared to the samples where tTS was absent.

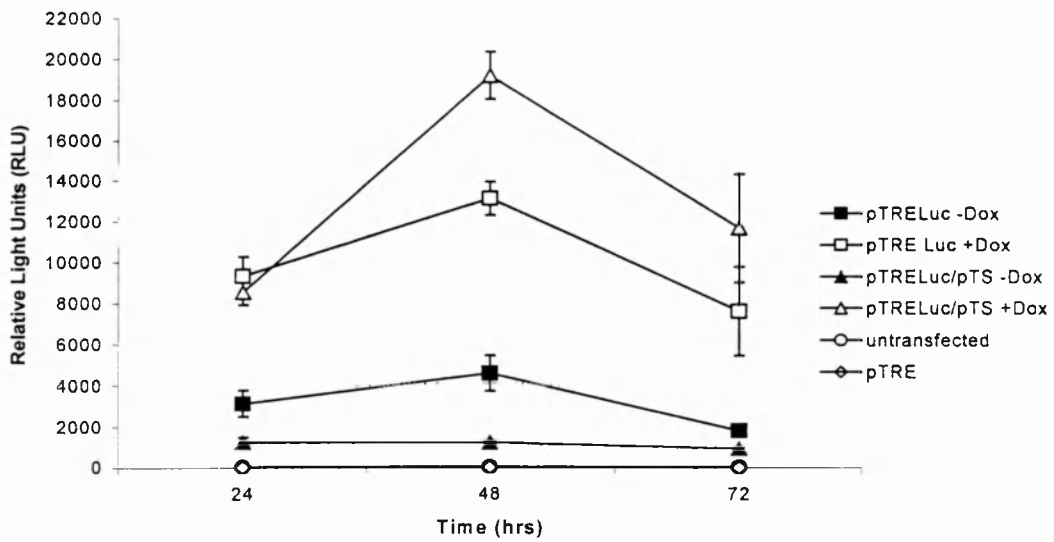


Figure 7.9: Expression of tTS reduces basal levels of gene expression in 293-On cells.
Protein from 293-On cells transfected as indicated was analysed for luciferase expression in a luciferase assay. + Dox: cells cultured in 1 μg/ml Dox; - Dox: cells cultured in 0 μg/ml Dox.

To investigate if the same effect could be obtained when the highly cytotoxic GALV gene was expressed, 293-On cells were transfected with pTRE-GALV alone or pTRE-GALV plus ptTS in the presence or absence of Dox. Cells were also transfected with pCR3.1-GALV as a positive control or pEGFP as a negative control. Fusion levels were monitored over 96 hrs and the results are summarised in **Table 7.5**. In the absence of Dox background levels of fusion were reproducibly reduced when cells were co-transfected with tTS and by 96 hrs only approximately 10% of the cells were forming syncytia, compared to approximately 80% of cells in the pTRE-GALV alone samples. Furthermore, levels of Dox-induced fusion in the tTS expressing cells were similar to those obtained in the positive control.

	Timepoints (hrs)			
Sample	24	48	72	96
pTRE-GALV	+	++	+++	++++
pTRE-GALV + ptTS - Dox	-	+	+	+
pTRE-GALV + ptTS + Dox	+++	++++	++++	+++++
pCR3.1-GALV	++++	+++++	+++++	+++++
pEGFP	-	-	-	-
untransfected	-	-	-	-

Table 7.5: tTS expression reduces background fusion in 293-On cells transiently transfected with pTRE-GALV.
293-On cells were transfected with indicated plasmid and syncytia formation was monitored over 96 hrs. + Dox: cells cultured in 1 μg/ml Dox; - Dox: cells cultured in 0 μg/ml Dox.
-: 0% fusion; +: 1-20%; ++: 21-40%; +++: 41-60%; ++++: 61-80%; +++++: 81-100%.

7.6 Development of a human melanoma cell line expressing GALV under the control of the Tet-On system.

Our results thus far demonstrate the feasibility of developing stable GALV-expressing tumour cell lines utilising the Tet-On system of transcriptional regulation. Based on these results, and the results of chapter 5 showing that FMG-mediated fusion

significantly enhances the immunogenicity of syngeneic/allogeneic vaccine we decided to develop a human melanoma cell line that would conditionally express GALV upon Dox stimulation. This cell line could serve as an "off the shelf" fusion partner to patient-derived material for the generation of fusing human tumour cell vaccines for clinical use. The human melanoma cell line A375M was sequentially modified as shown in **Figure 7.10** to express the key components of the regulation system.

A375M cells were initially transfected with the pTet-On plasmid alone or pTet-On plus ptTS and grown in 1 mg/ml Neomycin. Individual transformed colonies were isolated, further expanded and tested for basal levels of gene expression and inducibility of gene expression. A total of 33 out of 48 colonies initially picked from the plates survived the expansion process. The clones were transfected with pTRE-Luc in the presence or absence of 1μg/ml Dox and 48 hrs later luciferase expression levels were determined. Results from representative clones are shown in **Figure 7.11A**. In addition, the fold increase in the levels of luciferase expression in the presence of Dox compared to expression levels in the absence of Dox is shown in **Figure 7.11B**. Clones 2:14, 2:22 and 2:30, expressing both the pTet-On and ptTS plasmids, were chosen as the clones with the lowest basal level of gene expression and highest induction potential.

A375M-On clones 2:14, 2:22 and 2:30 were subsequently transfected with pTRE-GALV-Puro and grown in 0.4 μg/ml of Puro. As before, individual transformed colonies were isolated and expanded. A total of 60 sub-clones survived the expansion process and were screened for GALV expression and fusion induction in the presence of Dox. Results from representative clones are shown in **Table 7.6**. High levels of fusion could be induced in several sub-clones upon Dox treatment, all of which originated from A375M-On clone 2:14. By day 3 of Dox treatment, approximately 80% of cells of sub-clone 21 and approximately 60% of cells of sub-clones 12, 17 and 23 were forming syncytia. Syncytia formation reached very high levels by day 6 of Dox treatment, with 90-100% of cells of sub-clones 17 and 21 and 70-80% of cells of sub-clones 2, 6, 10, 12, 22 and 23 forming syncytia.

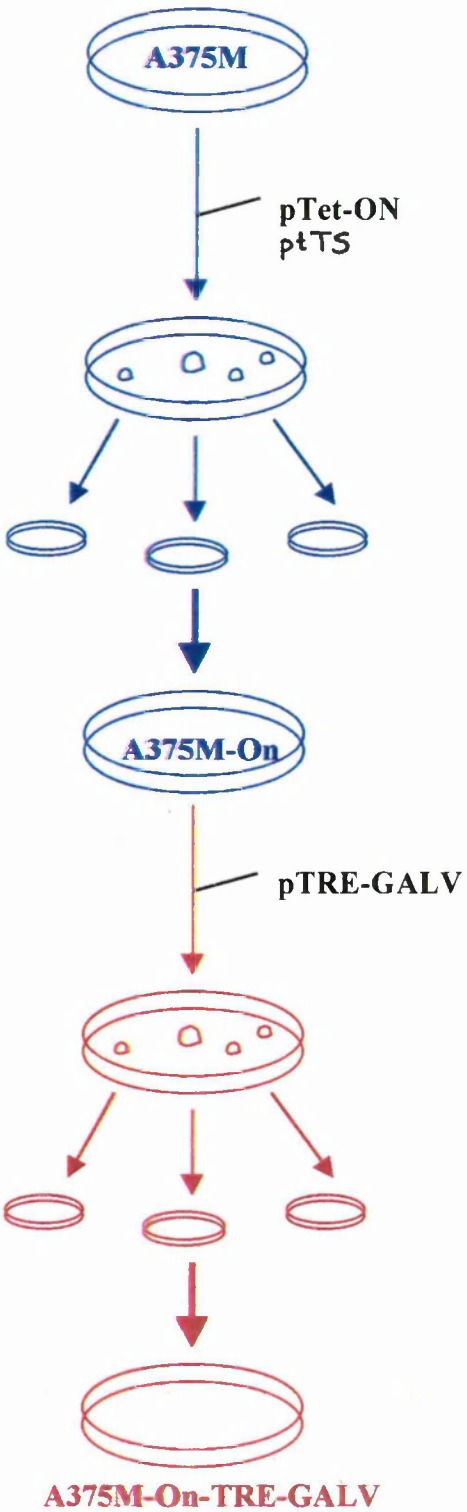


Figure 7.10: Outline of strategy used to generate double-stable A375M cells.

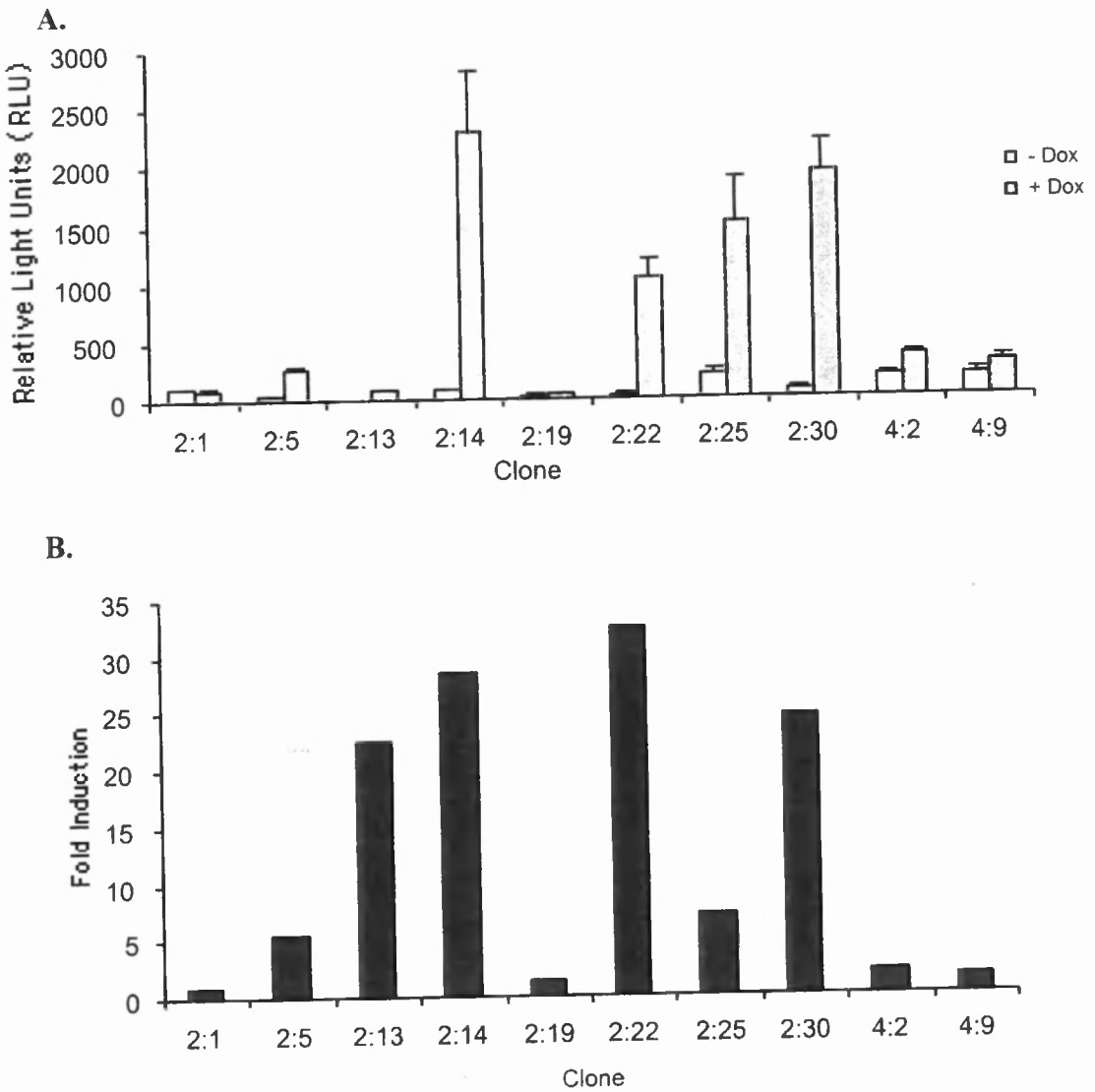


Figure 7.11: Screening of A375M-On clones for low basal gene expression and high induction potential.

A. A375M-On clones cultured in 0 or 1 μg/ml Dox were transfected with pRTELuc and 48 hrs later protein was extracted and analysed for luciferase activity. **B.** fold induction of luciferase expression in A375M-On clones.

A375M-ON clone	pTRE-Galv sub-clone	Day 3		Day 6	
		+ Dox	- Dox	+ Dox	-Dox
14	2	++	-	++++	-
	6	+	-	++++	-
	9	-	-	+	-
	10	+	-	++++	-
	12	+++	-	++++	-
	14	-	-	++	-
	17	+++	-	+++++	-
	21	++++	-	+++++	-
	22	++	-	++++	-
	23	+++	-	++++	-
22	21	+	-	-	-
	22	+	-	-	-
30	1	+	-	+	-
	3	+	-	+	-
	8	-	-	-	-
	9	-	-	-	-
	15	-	-	-	-
	20	+	-	+	-

Table 7.6: Induction of fusion in double-stable A375M-On sub-clones expressing GALV.

A375M-On-TRE-GALV double stable sub-clones were cultured in media containing 0 μg/ml Dox or 1 μg/ml Dox and extent of syncytia formation was assessed 3 and 6 days later. -: 0% fusion; +: 1-20%; ++: 21-40%; +++: 41-60%; ++++: 61-80%; +++++: 81-100%.

7.7 Summary

The Tet-On system for transcriptional regulation of gene expression was employed in these studies to regulate expression of the GALV envelope gene. Transient transfection of 293-On or Hela-On cells lead to high basal levels of gene expression as demonstrated by Luc or GALV expression in the absence of Dox. Following stable selection of transduced cells, 293-On and Hela-On cell lines could be developed that exhibited minimal to no basal levels of syncytia formation and high levels of Dox-induced fusion. Co-expression of tTS in the cells expressing the rtTA and TRE-GALV led to significant reduction of the levels of basal gene expression. These results demonstrate that it is possible to generate stably-transduced human cell lines expressing the GALV FMG under the control of Dox.

Based on the results shown in chapters 5 and 6 we decided to pursue the development of fusing tumour cell vaccines for therapeutic application in humans. "Off the shelf" fusing cell lines could be used to fuse with patient derived tumour material *ex vivo* and the fusing vaccine would be irradiated and administered to the patient. Alternatively, an "off-the-shelf" fusing tumour cell vaccine could be directly administered into a patients tumour nodule. Utilising the Tet-On system we were able to demonstrate the feasibility of generating conditionally fusing human melanoma cell lines. These cell lines expressed all three components of the Tet-On system, the rtTA, the TRE-GALV and the tTS, and expression of high levels of GALV was induced only following treatment with effective concentrations of Dox.

Chapter 8

Discussion

Employing immunotherapy strategies for the treatment of patients with cancer has gained significant respect over the last two decades. This has come as a direct result of advances in the understanding of the molecular basis of immunological responses to cancer, along with a better understanding of cellular immunology. The ultimate goal of cancer immunotherapy is the induction of a strong tumour-specific immune response against the primary tumour, as well as metastatic disease, ideally leading to the establishment of immune memory and prevention of further tumour recurrence. A hallmark in the evolution of cancer immunotherapy has been the realisation that tumour cells express a range of tumour associated antigens against which an immune response can be raised. Advances in the molecular characterisation of tumours led to the identification of numerous such TAA, including tissue-specific differentiation antigens, shared tumour-specific antigens and unique tumour antigens (Van den Eynde and Brichard 1995; Rosenberg 1997; Gilboa 1999). However, the majority of these antigens are centered around a limited selection of tumour types and the dominant immunogenic epitopes have been identified only for a restricted set of MHC haplotypes. This makes the generalised application of specialised TAA-based active specific immunisation for cancer treatment a limited approach for the majority of cancer types.

As an alternative approach tumour cell vaccines have been widely applied in animal models and in human studies, where the need for characterisation of individual TAA is obviated. First generation tumour cell based vaccines consisted primarily of irradiated autologous whole tumour cells or tumour cell lysates combined with non-specific adjuvants such as *BCG* or *C. parvum* (Nawrocki and Mackiewicz 1999; Schadendorf, Paschen et al. 2000; Moingeon 2001). The study of such vaccines in various experimental systems has demonstrated the feasibility of therapeutic anti-tumour vaccination in animal models. Early clinical trials in melanoma and renal cell cancer with such vaccines have demonstrated that it is possible to break immune tolerance to TAA and induce a specific anti-tumour immune response in human (McCune, O'Donnell et al. 1990; Hoover, Brandhorst et al. 1993; Greten and Jaffee

1999), although the overall response rates were low and occurred in patients with low tumour burden (similar to animal data).

Although autologous tumour cell vaccines offer the advantage of possessing all the relevant patient specific TAA against which an immune response needs to be raised, the large-scale generation of such individualised cell vaccines is currently limited by the difficulties in routinely and successfully establishing stable cell lines from biopsy material of patients tumours. This is particularly acute when genetic modification of the vaccine cells is desirable. An alternative approach which eliminates this difficulty is the use of long term established cell lines, called allogeneic cancer cells lines since they do not originate from the patient in which they are going to be used. The use of allogeneic cell lines for purposes of vaccination is feasible given that TAA are often shared among a large number of patients and antigens from tumour cells can be efficiently cross-presented by host APCs to cross-prime host T-cells (Mitchell, Kan-Mitchell et al. 1988; Huang, Golumbek et al. 1994; Ochsenbein, Klenerman et al. 1999). Allogeneic cell lines offer the great advantage that they can easily be further enriched with specific tumour antigens or genetically modified to express immunostimulatory molecules (see below).

Encouraging results on the efficacy of allogeneic vaccines have been obtained in the clinic. Morton and colleagues (Morton, Foshag et al. 1992) have developed a polyvalent melanoma cell vaccine (Cancer-Vax) developed from three human melanoma cell lines. This is an antigen-enriched vaccine containing at least 11 tumour-associated antigens found in melanoma and other tumours and 8 melanoma associated antigens (Chan and Morton 1998). In Phase II trials with stage IV melanoma patients, Cancer-Vax plus BCG yielded an overall response rate of 15-20%, significantly improved compared to historical controls. A randomised phase III trial is underway to confirm the effectiveness of the allogeneic vaccine to induce melanoma specific anti-tumour immune responses.

Another example of an allogeneic vaccine is the allogeneic vaccine developed by Mitchell and colleagues. Melacine consists of lysates from two mechanically disrupted human melanoma cells lines (Mitchell, Harel et al. 1993). Vaccination of patients with Melacine plus the adjuvant DETOX resulted in 20 objective responses

in 106 patients (Mitchell, Harel et al. 1993). However, a randomised phase III study of a lyophilised version of Melacine in 140 Stage IV melanoma patients failed to demonstrated enhanced clinical benefit of the vaccine when compared to four-drug standard chemotherapy, although significantly lower adverse effects were observed with the vaccine (Mitchell 1998).

Animal studies have shown that successful vaccination is intimately associated with the induction of host cellular immune responses, with CD8 +ve and CD4 +ve T cells playing a key role (Toes, Ossendorp et al. 1999). The immunogenicity of tumour cells used in the vaccines could be enhanced by genetic modification of the tumour cells to express immunostimulatory cytokines, co-stimulatory molecules, or MHC molecules (Nawrocki and Mackiewicz 1999; Bocchia, Bronte et al. 2000). Such genetic modifications have been shown to help overcome immune tolerance or anergy and mediate tumour rejection in both CD4 +ve T helper cell dependent and independent manner (Fearon, Pardoll et al. 1990; Baskar, Ostrand-Rosenberg et al. 1993).

8.1 Genetic Modification of Allogeneic Tumour Cell Vaccines with the Genes for IL-12, GM-CSF, IFN-γ or hsp70 Does Not Enhance the Immunogenicity of the Allogeneic Vaccines.

Cytokine gene modification of tumour cell vaccines has been a very popular approach for enhancing anti-tumour immune responses, partly due to the pleotropic mechanism of action of the majority of cytokines (Pardoll 1998; Mach and Dranoff 2000; Schadendorf, Paschen et al. 2000). Importantly, sustained release of cytokines in the local tumour environment mediates dramatic local inflammatory effects without any systemic or toxic effects often associated with systemic administration of cytokines. The aim of the first part of the thesis was to investigate the effect of cytokine modification on the immunogenicity of the allogeneic melanoma vaccine, K1735, and the induction of an anti-tumour immune response against the syngeneic and poorly immunogenic B16 melanoma.

GM-CSF is one of best studied cytokines in strategies of genetic modification of tumour cell vaccines. GM-CSF up-regulates surface expression of MHC and co-stimulatory molecules, stimulates macrophages and DCs and enhances the antigen presenting capacity of APCs (Dranoff, Jaffee et al. 1993; Chiodoni, Paglia et al. 1999). In combination these effects lead to enhanced T cell reactivity against tumour cells. GM-CSF has been shown to effectively generate systemic immunity against a number of poorly immunogenic tumours. The first study to show clear evidence of the effectiveness of GM-CSF modification was that of Dranoff et al who demonstrated that expression of GM-CSF by the very poorly immunogeneic B16 melanoma enhanced the immunogneicity of an autologous vaccine and lead to significant protection of mice from subsequent tumour challenges (Dranoff, Jaffee et al. 1993). Other studies in melanoma and other tumour models have shown similar results (Castleden, Chong et al. 1997; Ali, McLean et al. 2000; Kinoshita, Kono et al. 2001; Todryk, Birchall et al. 2001). In the clinic, vaccination of patients with metastatic melanoma with an irradiated autologous melanoma vaccine secreting human GM-CSF stimulated potent antitumour cellular and humoral immunity as demonstrated be dense infiltration of metastatic lesions with $CD4^+$ and $CD8^+$ T cells and plasma cells post vaccination, leading to significant destruction (at least 80%) of the tumour cells in the infiltrated metastasis, and enhanced IgG antimelanoma antibody production post vaccination (Soiffer, Lynch et al. 1998).

IL-12 is a critical element in the host defence against cancer and the mechanisms behind its potent anti-tumour effects involve components of both innate and adaptive immunity. It is produced by activated macrophages, DCs and B cells. It stimulates proliferation of NK cells and T cells and induces expression of a range of cytokines, promoting Th1 responses (Trinchieri 1995). IL-12 has been shown to promote the destruction of the tumour vasculature and the inhibition of angiogenesis (Voest, Kenyon et al. 1995) which may contribute to the direct anti-tumour effect of IL-12. Several pre-clinical studies have demonstrated the ability of IL-12 to induce potent local immune responses, to promote regression of pre-established tumours and to enhance systemic protective immunity against poorly immunogenic tumours (Brunda, Luistro et al. 1993; Tahara, Zitvogel et al. 1995; Brunda, Luistro et al. 1996; Hendrzak and Brunda 1996; Cavallo, Signorelli et al. 1997).

INF-γ plays a central role in tumour surveillance by rendering tumour cells more immunogenic (Kaplan, Shankaran et al. 1998). It upregulates surface expression of MHC class I and class II molecules, molecules involved in antigen processing, and co-stimulatory molecules thereby promoting antigen presentation, recognition and elimination by tumour-specific T cells (Boehm, Klamp et al. 1997; Kaplan, Shankaran et al. 1998). Furthermore, IFN-γ plays a key role in promoting innate antitumour immunity by recruiting and activating cells such as NK cells, macrophages and neutrophils, while it can have direct anti-tumour effects by inhibiting tumour angiogenesis (Qin and Blankenstein 2000). IFN-γ expression by tumour cells leads to potent and long-term antitumour immunity in mouse models (Gansbacher, Bannerji et al. 1990; Abdel-Wahab, Dar et al. 1996; Yang, Vervaert et al. 1999).

Based on these findings and on previous work in our laboratory and by others demonstrating the feasibility and value of using allogeneic tumour cell vaccines for cancer treatment (Kim, Russell et al. 1992; Thomas, Greten et al. 1998; Kayaga, Souberbielle et al. 1999; Melcher, Todryk et al. 1999; Chang, Chen et al. 2000; Kircheis, Kupcu et al. 2000; Habal, Gupta et al. 2001) we investigated the effect of modifying an allogeneic melanoma vaccine with the genes for GM-CSF, IL-12 or IFN-γ. We hypothesised as with syngeneic vaccines, modification of an allogeneic vaccine with these cytokine genes could significantly enhance the immunogenicity of the vaccine, leading to immunity against the autologous tumour. The model used in these studies was a well-established mouse melanoma model in which K1735 melanoma cells (H-2^k) (Kripke 1979), derived from C3H mice, were used as the allogeneic tumour cell vaccine in a model of protection against the development of B16 melanoma (H-2^b) (Fidler 1975), originating from C57BL/6 mice, in C57BL/6 mice (H-2^b). This serves as a very good model to investigate mechanisms to enhance the immunogenicity of a vaccine, since irradiated unmodified K1735 are very poor immunogens, leading to poor protection against a B16 challenge in C57BL/6 mice (**Figure 3.1**) (Knight, Souberbielle et al. 1996; Kayaga, Souberbielle et al. 1999; Melcher, Todryk et al. 1999; Todryk, Birchall et al. 2001). Despite the poor immunogenicity, K1735 cells can become effective vaccines under appropriate

immunological conditions (Kayaga, Souberbielle et al. 1999; Melcher, Todryk et al. 1999).

C57BL/6 mice were vaccinated with irradiated K1735 that had been previously modified to express murine-GM-CSF, -IL12 or –IFN-γ. Animals received 2-3 consecutive vaccinations 7 days apart and were then challenged with a lethal dose of live B16 cells. As shown in **Figures 3.2, 3.3** and **3.4** vaccination of mice with irradiated K1735-IL-12, K1735-GM-CSF or K1735-IFN-γ did not induce systemic immunity against the B16 tumour. This result was reproducible, suggesting that expression of IL-12, GM-CSF or IFN-γ could not enhance the immunising potential of irradiated K1735 cells under the conditions used. Although the dose of vaccine cells was not varied in these experiments to rule out the possibility that the vaccine dose was not sufficient to prime an immune response, previous experience in the laboratory had indicated that this dose was effective when combined with adoptively transferred DC (Melcher, Todryk et al. 1999). In addition, the dose used in our studies falls well within the range of K1735 vaccine doses published in the literature (Knight, Souberbielle et al. 1996; Kayaga, Souberbielle et al. 1999; Todryk, Birchall et al. 2001; Todryk, Birchall et al. 2001).

The level of cytokine release in the local microenvironment is a very important parameter in the effectiveness of cytokine-modified tumour cell vaccines. Indeed, Chang et al. have demonstrated that the success of their GM-CSF-secreting antigen specific allogeneic vaccine in inducing systemic protective immunity against a lethal tumour challenge was proportional to the GM-CSF level secreted by the allogeneic tumour cell vaccine, with cells releasing higher levels (~200 ng/10^6 cells/24 hrs) being far more effective in inducing antitumour immunity than cells releasing lower levels (~60 ng/10^6 cells/24 hrs) (Chang, Chen et al. 2000). This suggests that there may be a minimum cytokine secretion level necessary for a tumour cell-based vaccine to efficiently induce antitumour immunity. Similarly, Kayaga et al. (Kayaga, Souberbielle et al. 1999) have found in their studies that their irradiated B16-GM-CSF vaccine was ineffective in protecting mice from a 3 day established B16 tumour, in direct contrast to the findings of Dranoff et al. (Dranoff, Jaffee et al. 1993) who demonstrated that an irradiated B16-GM-CSF vaccine was capable of protecting

100% of the animals from pre-established B16 tumours in a similar vaccination protocol. One possible explanation for this dramatic difference could be the difference in the level of GM-CSF secreted by the tumour cell-based vaccine. In the former study GM-CSF secretion was much lower compared to that secreted by the tumour cell-based vaccine in the later study (32.75 ng/10^6 cells/48 hrs compared to 300 ng/10^6 cells/48 hrs). It is challenging to compare the levels of cytokine secreted by cytokine-modified tumour cell-based vaccines in the different studies and determine an effective level of cytokine secretion as most studies use different tumour models and different dose and vaccination regiments. In the studies presented in this thesis the modified K1735 cells secreted relatively low levels of cytokines, with K1735-IL-12 cells secreting 8.3 ng/4x10^5 cells/48 hrs of IL-12, the K1735-GM-CSF cells secreting 1.4 ng/4x10^5 cells/48 hrs and the K1735-IFN-γ secreting 3.7 ng/4x10^5 cells/48 hrs. Although in the lower range, these levels are comparable to other published levels for autologous or allogeneic cytokine modified tumour cell-based vaccines (Chong, Todryk et al. 1998; Dunussi-Joannopoulos, Runyon et al. 1999). However, lack of effectiveness of our cytokine-modified allogeneic vaccines could be due to the relatively low levels of cytokine secretion.

Irradiation of cytokine secreting vaccine cells has been shown to have an effect on the levels of cytokine released by the vaccine cells (Ali, McLean et al. 2000) and on their efficacy (Simova, Bubenik et al. 1998). In our hands, irradiation of the K1735-IL-12, -GM-CSF or –IFN-γ vaccine cells had no significant effect on the production and release of any of the three cytokines by the modified K1735 cells as shown in **Figure 3.5**. The levels of IL-12 and GM-CSF release remained constant following irradiation and were comparable to those obtained by the non-irradiated K1735-IL-12 and K1735-GM-CSF cells respectively. Release of IFN-γ, although lower compared to that obtained by live cells, remained constant following irradiation.

These data showing a lack of efficacy of cytokine modified K1735 vaccines are in agreement with recent literature. Specifically, using a tumour model very similar to the one used in this thesis Todryk et al. have recently demonstrated that the efficacy of cytokine modified K1735 vaccines varies significantly depending on the model used (Todryk, Birchall et al. 2001). When a K1735-GM-CSF vaccine was used as an

allogeneic vaccine to immunise against a B16 tumour challenge in a protective vaccination model in C57BL/6 mice the efficacy was the same as the unmodified parental vaccine. In contrast, when used as a syngeneic vaccine in C3H mice to immunise against a K1735 tumour challenge significantly better protection was observed compared to K1735 alone. The authors suggested that although an immune response was occurring against the allogeneic K1735 cells that could potentially lead to cross-priming of allogeneic tumour cell-derived antigens by the host APC, cytokine secretion by the allogeneic cells could not further enhance the allo MHC-induced immune response.

In another study by Kircheis et al. (Kircheis, Kupcu et al. 2000) K1735-IL-2 or B16-IL-2 modified irradiated allogeneic vaccines were capable of eliciting significant immunity and cross-protect DBA/2 mice from an M-3 syngeneic melanoma ($H\text{-}2^d$) challenge. However, when K1735-IL-2 or M3-IL-2 allogeneic vaccines were used to immunise against the syngeneic B16F10 melanoma in C57BL/6 mice no cross-protection was observed. Similar observations were made by these authors with a K1735-GMCSF allogeneic vaccine in the C57BL/6 model.

Expression of allogeneic MHC on the surface of an allogeneic vaccine leads to immune stimulation, "rejection" and rapid clearance of the allogeneic cells as shown in chapter 4. We hypothesise that combination of immunostimulatory cytokines such as IL-12, GM-CSF or IFN-γ, with a potent allo-response will lead to considerably faster elimination of the vaccine cells, not allowing enough time for efficient immune stimulation. Therefore, this could be a contributing factor to the failure of our cytokine-modified K1735 vaccines.

Hsp70, a member of the highly conserved family of heat shock proteins, has been shown to enhance the immunogenicity and consequently vaccine potency of autologous B16 melanoma cells when constitutively overexpressed (Todryk, Melcher et al. 1999). Hsp70 over-expression by B16 tumour cells is associated with increased immune cell infiltration at the tumour site, a TH1 profile of intratumoural cytokine production and enhanced antigen uptake by DC (Melcher, Todryk et al. 1998; Todryk, Melcher et al. 1999). Furthermore, hsp70 has been shown to play an important role in

the priming of efficient anti-tumour immune responses via its ability to chaperone tumour antigens to subsets of APCs (Blachere, Li et al. 1997; Castellino, Boucher et al. 2000). We therefore looked at the ability of hsp70 to enhance the immunogenicity of the allogeneic K1735 cell vaccine in our allogeneic prophylactic vaccination model. Overexpression of hsp70 was achieved by either genetic modification of the tumour cells to constitutively overexpress hsp70 or by various conditions of heat shock, which we show lead to overexpression of hsp70 (**Figure 3.6**). Vaccination of C57BL/6 mice with irradiated K1735-hsp70 cells however did not lead to enhanced protection of the animals from subsequent B16 tumour challenge, suggesting that hsp70 overexpression had no significant effect on the immunogenicity of the allogeneic K1735 cells. We hypothesise that similar to cytokine modified allogeneic cells, hsp70 modified allogeneic cells are cleared by the immune system too quickly and presumably in a non specific manner such that hsp70 modification is not effective in enhancing any antigen-specific immune responses.

Great variation exists between the models of allogeneic tumour cell-based vaccination in the mouse system and this most certainly accounts to a great extent for the significant variation observed in the effectiveness of allogeneic vaccination. Several studies of allogeneic vaccination for melanoma have utilised the K1735/B16 model in C57BL/6 mice. This model described by Knight et al. (Knight, Souberbielle et al. 1996) is one of the few models where completely allogeneic tumour cells (K1735-M2) carrying only the natural tumour antigens have been shown to induced cross-protection (although very weak) against an autologous tumour (B16-F10). This cross-protection could be further enhanced by transfection of the allogeneic cells with the GM-CSF gene (Kayaga, Souberbielle et al. 1999). The data in chapter 3 as well as data by Todryk et al. (Todryk, Birchall et al. 2001) and Kircheis et al. (Kircheis, Kupcu et al. 2000) however demonstrate that variation in the effectiveness of allogeneic vaccines exists even when the same model of study is used. The diversity of the different K1735 sublines used in these studies have probably contributed to this effect. Furthermore, differences in the *in vitro* manipulation of the same cell line may lead to changes in its characteristics, such as antigen expression and surface MHC expression, and this may significantly affect its immunogenic characteristics. To obtain a better understanding of the cell lines used as the basis of the allogeneic vaccines in our studies we characterised the immunological features of the K1735 as

well as the B16 cell lines used. Several tumour markers were studied including TAA expression, MHC expression and costimulatory molecule expression.

One of the most important parameters for the induction of cross-protection by allogeneic vaccines is the expression of TAA by the allogeneic cells that are present in the target tumour. In human melanoma a number of the different melanoma–associated antigens identified thus far are known to be shared by more than 50% of melanoma patients. In murine melanoma however, little information is known about the expression of immunologically relevant melanoma-derived tumour antigens or the frequency of shared expression of such potential tumour rejection antigens between the different melanoma cell lines. To address these issues in our model system we screened the K1735 and B16 cell lines for TAA expression using rtPCR. The analysis showed that of the eight melanoma antigens tested, only four were shared between the two cell lines (**Table 4.1**). Although expression of a wider panel of melanoma antigens could be tested, the data suggest that a relative paucity of shared antigens exists between the two cell lines used in our vaccination system and this may contribute to the poor vaccine efficacy. However, it is important to mention that K1735 express TRP-2 and gp100, two known tumour rejection antigens for B16 *in vivo*, although at a lower level than B16 (Zhai, Yang et al. 1996; Bloom, Perry-Lalley et al. 1997). Our data are in some agreement with the findings of Peter et al. (Peter, Mezzacasa et al. 2001) who showed a limited antigen match between K1735 and B16. Shared expression of relevant tumour antigens between the allogeneic vaccine and the autologous tumour alone, however, is probably not sufficient to induce effective cross-protection. This is best demonstrated in the study of Kircheis et al. (Kircheis, Kupcu et al. 2000) where the efficacy of cross-protection generated between two melanoma cell lines, M3 and B16, varied depending on the mouse model used. Their studies suggested that the MHC content of the host may play an important role in the effective presentation of relevant antigenic epitopes. Antigens deriving from one cell line may not be suitable for presentation by the MHC molecules of the host APC and therefore may not be exploited for tumour rejection by the host.

Surface expression of MHC molecules by the two melanoma cell lines was tested using surface staining with antibodies specific for MHC Class I or Class II and cytofluorometric analysis (Figure 4.1). K1735 cells expressed moderate to high levels

of the MHC class I molecules tested (H-2D^k and H-2K^k) while B16 cells did not express any detectable levels of H-2D^b or H-2K^b under the experimental conditions used (**Figure 4.1A**). This high surface expression of MHC class I may contribute to a more rapid clearance of the K1735 when used as an allogeneic vaccine. As expected, none of the two tumour cell lines expressed any of the MHC Class II molecules tested (**Figure 4.1B**).

Subcutaneous injection of irradiated K1735 cells in C57BL/6 mice led to the generation of a proinflammatory environment at the injection site, with high levels of IFN-γ and TNF-α detected by rtPCR analysis of the injection site at 24 hrs and 48 hrs post injection respectively (**Figure 4.3**). No IL-10 expression was detected up to 4 days post injection. Induction of a proinflammatory local environment correlated well with the clearance of the K1735 cells from the injection site (**Figure 4.3**), suggesting that clearance of the allogeneic cells from the injection site was mediated by activated infiltrating immune cells. The presence of IFN-γ in the modified allovaccine site may potentially explain the lack of effect of adding further IFN-γ, or the IFN-γ inducer IL-12, to the vaccine site via gene modification of the allovaccine. To further understand the mechanism of clearance of the allogeneic vaccine cells the injection sites were analysed for the types of infiltrating immune cells. Analysis of the data showed that by 48 hrs post injection K1735 injection sites were heavily infiltrated by NK cells, macrophages and granulocytes. The infiltration of the allogeneic cell injection sites was significantly higher than that observed in syngeneic B16 cell injection sites. Although a proinflammatory environment was created following injection of irradiated allogeneic cells, the infiltrate into K1735 may reflect clearance of the allogeneic tumour cells via non-specific effector cells, such as NK cells. It appears that this clearance occurs without generating a specific memory T cell response to the tumour antigens, such that subsequent rechallenge with antigen-expressing B16 cells is not affected. Together these data showing inflammation and cell infiltration to the unmodified vaccine site may explain why modifying the vaccine environment with the molecules described in Chapter 3 did not enhance allo-vaccination.

8.2 VSVG-Induced Fusogenic Cell Death Is Highly Immunogenic and Enhances the Efficacy of a K1735/B16 Vaccine.

Previously in the lab we had described a novel class of therapeutic genes for the control of tumour growth (Bateman, Bullough et al. 2000; Diaz, Bateman et al. 2000). Expression of genes encoding viral FMGs in tumour cells leads to very potent local cell killing, with fusion of neighboring tumour cells to each other and the formation of large multinucleated syncytia. Syncytia formation is associated with subsequent nonapoptotic, autophagic-like mechanisms of death that are immune potentiating (Bateman, Harrington et al. 2002) through the induction of stress related proteins and expression of the viral immunogen that could act as potent adjuvants (Melcher, Todryk et al. 1998; Bateman, Bullough et al. 2000; Gough, Melcher et al. 2001). We therefore decided to investigate whether FMG-induced fusion with all its immunological consequences would provide an effective method to liberate relevant tumour antigens from allogeneic cells and additional immune-potentiating signals sufficient to stimulate an effective antitumour immune response. Once again the K1735 allogeneic melanoma vaccination model was studied against the development of B16 melanoma in C57BL/6 mice. To induce fusion in murine tumour cells a novel FMG gene, VSV-G, was characterised and utilised. Phosphotidylserine is the receptor for VSV-G expressed widely on the surface of murine cells in abundant levels. As mentioned previously (Introduction and Chapter 5), VSV-G is nonfusogenic in normal ambient pH (approximately pH 7.5). Fusion can only be induced when the ambient pH reaches pH 5.5-5.7. This condition of acidic environment triggers a conformational change in the VSV-G molecule that converts the protein into a fusogenic conformation (Fredericksen and Whitt 1995). VSV-G transfection of murine tumour cells, followed 24 hrs later by a transient pH drop, leads to extensive syncytia formation by 24-48 hrs following the pH drop (**Figure 5.1**).

To investigate the effect of VSV-G-induced fusion on the immunogenicity of allogeneic or syngeneic tumour cell vaccines K1735 or B16 fusing tumour cell vaccines were prepared and used to vaccinate mice in a prophylactic vaccination protocol. To control for the adjuvant effect expression of VSV-G on vaccine cells may have and also to control for the effects of the transient pH drop we used a mutant

VSV-G (VSV-G*) in which a single amino acid mutation reduces the amount of cell fusion induced by gene expression and pH drop by >90% (**Figure 5.1**) (Fredericksen and Whitt 1995). Mice were vaccinated weekly with 2-3 consecutive vaccinations of irradiated fusing tumour cells and approximately a week after the last vaccination they were challenged with live B16 tumour cells as before. Fusion of K1735 cells generated no statistically significant therapeutic benefit relative to K1735 cells transfected with VSV-G* or naïve mice (**Figure 5.2B**). Fusion of B16 cells to each other was only moderately a more effective vaccine than irradiated B16 cells (**Figure 5.2A**).

Several groups have investigated the use of allogeneic/autologous vaccine preparations as therapeutic vaccines for cancer with significant success. Staib et al. have demonstrated that a mixture of autologous B16-derived melanoma cells with allogeneic S91 melanoma cells was able to vaccinate mice against growth of cerebral B16 metastasis in C57BL/6 mice (Staib, Harel et al. 1993; Staib, Harel et al. 2001). This vaccine required IFN-γ-mediated up-regulation of Class I molecules on the vaccine cells, as well as the co-administration of the non-specific adjuvant, to be most effective. In our hands vaccines composed of mixtures of allogeneic and autologous melanoma cells induced no significant therapeutic effect against tumour growth (**Figure 4.7**) regardless of the relative proportion of allogeneic or autologous cells in the vaccine preparations. Several studies in the literature have demonstrated the use of semi-allogeneic or even semi-xenogeneic hybrid cells for generating potent antitumour vaccines (Kim 1979; Payelle, Poupon et al. 1981; Toffaletti, Darrow et al. 1983; Newton, Romano et al. 2000; Newton, Acierno et al. 2001). Enhanced immunity in these studies was probably a result of an association of TAA with allogeneic molecules, while TAA and alloantigens needed to be on the same cell for effective vaccination.

Hybrid cells between tumour cells in the literature have thus far been produced mainly using polyethylene glycol-mediated fusion and subsequent *in vitro* selection of the fused cells in appropriate selection media. However this approach is time consuming and relatively inefficient. We therefore investigated the use of FMG as a novel and very efficient mechanism to generate allogeneic/autologous tumour cell

hybrids and to determine the potency of such a vaccine in inducing protective antitumour immunity. FMG expression in a population of cells expressing the appropriate receptor for the FMG leads to very efficient and rapid generation of fused multinucleated cells (**Figure 5.1** and (Bateman, Bullough et al. 2000; Galanis, Bateman et al. 2001)). We hypothesised that a combination of expression of relevant TAA, allogeneic MHC and other alloantigens, immunogenic cell death associated with syncytia formation and the potential adjuvant effect of FMGs in the same vaccine may generate the appropriate environment for the induction of short term rejection of tumour, as well as long term T-cell mediated responses leading to protection of mice from a subsequent tumour rechallenge. K1735/B16 fusing tumour cell vaccines were generated by mixing each melanoma cell line at a 1:1 ratio, transfecting the mixed population with the VSV-G gene and inducing fusion by a transient pH drop. Mice were vaccinated with irradiated K1735/B16 fusing cells in a prophylactic vaccination protocol. Vaccination was very effective at protecting mice from a subsequent B16 tumour challenge with up to 80% of mice surviving rechallenge long term (>60 days), depending on the individual experiment (**Figure 5.3**). The range of protection observed in the experiments carried out with the K1735/B16-VSVG vaccine was from 30 to 80% of the animals in the treatment group. Overall the experiment was carried out seven times and 30% protection was obtained only once, while protection in the remaining experiments was consistently between 70 an 80%. These effects were not solely due to the adjuvant effect of the VSV-G immunogen, or vector-related components, or the effects of the transient 2 min pH drop, as a K1735/B16 vaccine expressing the fusion defective VSV-G (K1736/B16-VSVG*) (and exposed to the short drop in pH) gave only small therapeutic gains.

The mechanism by which tumour cells are fused (and subsequently die) seems to be a critical factor in the efficacy of the fusing allogeneic/syngeneic tumour cell vaccines, as demonstrated by the lack of efficacy of a PEG fused K1735/B16 vaccine (**Figure 5.4**). K1735/B16 cells were fused using the standard PEG-mediated fusion protocols, which generate small, disorganised clumping aggregates rather than large, organised syncytial structures that are characteristic of VSV-G fusion. Although further experiments are required to characterise the two different mechanisms of fusion of the K1735/B16 vaccine cells (i.e. level of fusion and induction of stress response signals),

the significant difference observed in the *in vivo* efficacy between the two different fusion preparations suggests that the mechanisms of cell fusion and its consequences are key factors determining the potency of a fusing semiallogeneic vaccine.

The irradiated K1735/B16-VSVG fusing vaccine was very effective in a therapeutic vaccination model where up to 80% of mice were cured of small established B16 tumours (**Figure 5.5**). Subsequent rechallenge of animals cured from the initial B16 tumour demonstrated that the fusing K1735/B16-VSVG vaccine was able to generate immunity which was long term and tumour specific (**Table 5.1**). Repetition of the experiment in athymic nude mice showed that the antitumour immunity was T cell dependent (Figure 5.6).

To investigate the precise contribution of allogeneicity compared to fusion we designed prophylactic vaccination experiments similar to the ones described above using B16 variants expressing either no MHC class I molecules or a single type of syngeneic or allogeneic MHC class I (Thomas, Greten et al. 1998). These data demonstrated that vaccines in which VSV-G induced fusogenic cell death was combined with allogeneic MHC and relevant tumour antigens were most potently immunogenic and induced long term protection (>60 days) of mice against syngeneic tumour growth (**Figure 6.3**). Furthermore, the protective immunity provided by fusing B16 MHC class I loss variant (B16neg-VSVG) and the B16 expressing a single type of MHC class I molecule (H-2K^b) (B16syng-VSVG) was similar (approximately 50% of the mice were protected long term from tumour challenge), suggesting that the anti tumour immune response is a result of cross priming of the relevant TAA by host APC and not a result of direct priming by the vaccine cells.

In the experiments described in this section of the thesis, a single gene modification, VSV-G-induced tumour cell fusion, is effective in generating both rejection of established tumour as well as long term T cell mediated protection responses. Considering that the immunological mechanisms involved in generation of short-term therapy may be different from those involved in long term protection (Prehn 1993; Castleden, Chong et al. 1997; Melcher, Todryk et al. 1998), syncytia of K1735 and B16 cells are likely to have several immunological consequences. The adjuvant effects of allo-reactivity, as well as the immunogenicity of the VSV-G antigen, are

likely to enhance the potency of fusing cell vaccines, by making them good targets for NK and other non-specific immune effector killing mechanisms. This is demonstrated to some extent by the analysis of the immune infiltrates at injection sites of irradiated K1735 or B16 cells which showed that the allogeneic cells induce a higher non-specific immune infiltration compared to the autologous cells (**Figure 4.6**). Furthermore, similar examination of injection sites of irradiated VSV-G expressing K1735/B16 vaccines showed that the presence of the VSV-G immunogen in the absence of any fusion in a vaccine preparation adds, although to a limited extent, to the observed infiltrate compared to tumour cells alone. However the effects of cell fusion clearly add significant immunological potency to the efficacy of the fusing vaccines over and above that provided by the adjuvant effects of allo- or anti-VSV-G reactivity alone. This is reflected very well in the enhanced recruitment of NK cells, macrophages, neutrophils and T cells, as well as in the levels of protection seen *in vivo* with fusing K1735/B16-VSVG vaccines compared to the those obtained with the non-fusing K1735/B16-VSVG* vaccines.

In addition, direct syncytial-mediated cell killing will also release immune stimulatory molecules at the vaccine site which will recruit and activate host APC (Gallucci, Lolkema et al. 1999; Todryk, Melcher et al. 1999), and stress proteins expressed within the syncytia (Bateman, Bullough et al. 2000) contribute directly to immune stimulation (Melcher, Todryk et al. 1998; Basu, Binder et al. 2000; Gough, Melcher et al. 2001). Based on previous work in the laboratory demonstrating the importance of macrophages in co-ordinating immune responses to tumour cell killing (Gough, Melcher et al. 2001) and in view of the observations *in vivo* of activation of a macrophage response to allogeneic cell vaccines (**Figure 4.6**) as well as VSV-G-fused semi-allogeneic vaccines (**Figure 6.1**), we hypothesised that macrophages are important mediators of the response to fusing cell killing. *In vitro* experiments demonstrated that macrophages can phagocytose tumour material released from dying syncytia of K1735/B16 cells much more efficiently than that released from K1735/B16 cell mixtures or entirely syngeneic B16-VSV-G fusing cell preparations (**Figure 6.5**), and that these macrophages are activated by allofusion (represented by TNF-α secretion, **Figure 6.5**) support our hypothesis. Taken together these data show that macrophages and dendritic cells can phagocytose tumour material released

from dying syncytia. Furthermore, they show that macrophages (but interestingly not dendritic cells) can sense allogenicity within fusing mixtures of autologous and allogeneic cells which stimulates both phagocytic uptake of antigens and activation of macrophage of TNF-α secretion. Therefore, macrophages recruited to the subcutaneous site of allogeneic cell-containing vaccination (as seen in **Figure 6.5**) will be significantly more activated *in situ* in the presence of fusing cells, permitting initiation of a more effective anti tumour immune response. Furthermore, experiments utilising a model tumour antigen, OVA, showed that syncytial cell death, but not other forms of cell death, promotes cross presentation by immature DC (**Figure 6.6**). Similar results have been obtained in experiments using a human tumour antigen, gp100, and human dendritic cells, demonstrating the relevance of the results described here in the context of a known melanoma antigen in the human system. Enhanced cross-presentation may be mediated by stress proteins such as heat shock proteins released by the dying fusing cells that are able to potentiate antigen uptake by DCs (Tamura, Peng et al. 1997; Castellino, Boucher et al. 2000). Other routes for antigen uptake from dying tumour cells and subsequent cross-presentation by DCs have also been reported that rely upon levels and routes of antigen release (Kurts, Miller et al. 1998; Mitchell, Nair et al. 1998).

Further investigation of the mechanism of cell death in syncytial structures has shown that syncytia-associated cell death occurs through pathways that lack the morphological, cytogenetic or biochemical markers of classical apoptosis (Bateman, Harrington et al. 2002). This is an important observation as it further demonstrates that fusogenic cell death is highly immunogenic as it is likely associated with activation of stress response programs (characteristic of non-apoptotic cell death) that led to efficient activation of anti tumour immune responses (Melcher, Gough et al. 1999; Todryk, Melcher et al. 1999; Basu, Binder et al. 2000; Gough, Melcher et al. 2001).

Overall, we hypothesise that the VSV-G-mediated syncitial death, in the presence of allo MHC, may resemble a highly pathogenic event. The simultaneous release of cell material from non-apoptotic death, while immune cells are non-specifically

stimulated by foreign MHC-peptide structures, would thus be a highly immunologically relevant event (Matzinger 1994).

8.3 Generation of FMG Gene Modified Human Melanoma Allogeneic Cell Lines Can Be Successfully Attained Using a Small Drug Inducible System for the Regulation of Gene Expression.

Clinically, we believe that the data presented in Chapters 5 and 6 are very significant for the design of tumour cell vaccines. By modifying either patient recovered tumour explants, or established allogeneic cell lines, with a gene for a FMG, fusing tumour cell vaccines can be produced *in vitro* for patient use. In addition, as a more direct translation of the observations of Chapter 5, a stable allogeneic cell vaccine could be engineered to undergo FMG-mediated fusion which could be boosted by the addition of patient-derived tumour cells prepared over a very short period of time following surgery to obtain the allogeneic/autologous mix that was shown to be most effective in our studies. In this respect the last chapter of this thesis focuses on optimising a method that could be employed to generate stable human FMG-expressing cell lines. FMGs are highly cytotoxic genes and, as expected, expression of these genes under standard constitutive promoters, such as CMV, in any cell line that expresses the appropriate FMG receptor would lead to uncontrolled syncytia formation and fusogenic cell death in a very short period of time. To obtain cell lines in which initiation of fusion is a tightly controlled event, which can reproducibly yield extensive syncytia formation, a gene expression regulation mechanisms is required, that will allow effective silencing of gene expression as well as very potent and immediate induction of gene expression. Such a gene regulation switch can be offered by the Tet-On transcription regulation system (Gossen, Freundlieb et al. 1995; Baron, Gossen et al. 1997; Rossi and Blau 1998), which is a bi-component system composed of the tetracycline-controlled transactivator (rtTA) and the tetracycline-responsive expression cassette (pTRE). Utilising this system we were able to demonstrate that expression of the very potent GALV gene in human cell lines can be controlled and subsequently stable cell lines expressing this gene could be generated.

Initial experiments utilising two human cell lines which are stably transduced with rtTA (293-On and Hela-On) and have the potential of high levels of gene expression upon Dox stimulation, demonstrated that at the transient transfection level expression of GALV under the control of the TRE promoter was leaky (**Table 7.1**; **Figure 7.4**; **Figure 7.5**) leading to significant fusion in the absence of any Dox. Similar leakyness in gene expression was also observed using a non-toxic gene, luciferase, suggesting that the basal gene expression in this system was not a function of the toxicity or the strong bystander effect of the GALV gene. Several reports in the literature have indicated the existance of background levels of gene expression using the Tet-On system, particularly when polyclonal pools of cells are studied. For example Bohl et al. (Bohl, Naffakh et al. 1997) studied the control of production of the therapeutic protein erythropoetin (EPO) in mice by transplanting engineered primary myoblasts. Using two retroviruses, one encoding rtTA downstream of a skeletal-muscle-specific promoter, and the other encoding mouse EPO under the TRE the authors modified mouse primary myogenic cells and following selection of a polyclonal population of transduced cells found that introduction of the TRE-EPO containing vector alone led to low-level EPO secretion that was further increased three-fold by adding the rtTA vector in the absence of Dox. This observation demonstrated that in the polyclonal pool studied the TRE-EPO promoter had some basal activity and rtTA activated to an extent irrespective of Dox induction. Addition of Dox led to a significant, 70 fold, induction of EPO secretion by transduced myoblasts *in vitro*. Background levels of gene expression in the absence of induction has been observed with several other inducible systems (Clackson 1997). Intrinsic effects, such as retention of the active form in the absence of inducing agent, and/or the effect of the integration site on the activity of the basal promoter of the target gene (position effect) are likely to play a significant role in the generation of the basal activity. The observations that basal expression can be detected even in the absence of the appropriate transactivator molecules (Bohl, Naffakh et al. 1997), and that stable cell clones can be obtained that exhibit very low background expression and high induction ratios, suggest that position effects are probably the major contributing factor to the variations observed in different experiments. In the case of expression of toxic genes such as the FMGs, where background levels of transcription in the uninduced state need to be negligible, optimisation of basal promoters will be

essential to obtain a stringent system of gene expression regulation (Hoffmann, Villalba et al. 1997).

Single cell selection for generation of cell lines exhibiting only very low basal gene expression is a strategy that has been employed by the majority of researchers utilising the Tet-inducible gene expression regulation system. In our studies we were able to select several 293-On and Hela-On subclones transduced with both rtTA and TRE-GALV. Screening of these double-stable cell lines for GALV expression in the presence or absence of 1 μg/ml Dox by assessing extent of syncytia formation using light microscopy revealed several subclones that exhibited no or very minimal background fusion in the absence of the inducer, Dox, and high levels of syncytia formation following the addition of the inducer (**Table 7.2 & 7.4**). GALV expression levels in the presence or absence of the inducer were confirmed by Northern Blot in the case of the Hela-On subclones.

Incorporation of a third component, the transcriptional silencer – tTS, in the Tet-On system has also been shown to significantly improve the performance of the system by reducing the basal level of expression without affecting the fully induced level (Freundlieb, Schirra-Muller et al. 1999). tTS has the reverse DNA binding properties from those of rtTA, i.e. it can bind the TRE in the absence of Dox and can therefore be used in the presence of the rtTA to actively suppress gene expression from the TRE-containing promoter without affecting rtTA function. Very importantly, rtTA and tTS have been designed such that they bear different dimerisation domains, preventing heterodimer formation between the two molecules. In transient expression experiments transfection of 293-On with pTRE-Luc and ptTS led to significantly lower basal levels of luciferase expression compared to that obtained in cells transfected with just the pTRE-Luc vector (**Figure 7.9**). Addition of Dox in the double transfected cells led to a 10-fold induction of luciferase expression by 48 hrs. Similar observations were made when GALV expression was tested in a similar experiment. Background fusion was significantly reduced when the tTS was co-expressed in the cells (**Table 7.5**). In addition, fusion of the polyclonal pools in the presence of the inducer appeared to be induced more rapid and to a higher extent. These observations demonstrate that for the purposes of our studies (generation of cell

lines expressing the highly toxic FMG genes) the tTS would be an essential component that can significantly improve our system.

The data obtained thus far demonstrated that stable GALV-expressing cell lines could be obtained utilising the Tet-On system of trancriptional regulation. We therefore decided to develop a human melanoma cell line which would conditionally express GALV and which could be used as an allogeneic fusion partner in the generation of allogeneic/autologous fusing tumour cell vaccines for use in human systems. Very few allogeneic melanoma cell lines exist that have been approved by the FDA for use in clinical trials in patients with melanoma. One example of an extensively studied vaccine is CancerVax, an antigen enriched polyvalant vaccine created by Morton and colleagues (Morton, Foshag et al. 1992). Access to this or other approved allogeneic human melanoma cell lines for the purposes of our studies was not possible during the time-course of this thesis. We therefore decided to use a widely studied human melanoma cell line available in the laboratory. A375 is a human melanoma cell line established from a female melanoma patient (Giard, Aaronson et al. 1973) and expresses some of the known melanoma antigens (Cormier, Hijazi et al. 1998). Following sequential transfection of this cell line with plasmids carrying rtTA, tTS and TRE-GALV and extensive *in vitro* screening of over 90 clones at least 8 stably transfected subclones could be obtained in which no background fusion could be detected while very high levels of fusion could be induced upon addition of Dox (**Table 7.6**). These GALV expressing human melanoma cell lines provide a very useful tool for the generation of fusing hybrid melanoma vaccines. Although extensive characterisation of these cells lines would be vital prior to any clinical use, they can potentially form the platform of a vaccine which can be further enriched with fusion of other allogeneic melanoma cells or patient-derived autologous melanoma cells. This would lead to the generation of potentially potently immunogenic polyvalant fusing melanoma cell vaccines for use in human studies.

One very important observation as far as the technical aspect of generating all of these stable cell lines mentioned thus far is concerned has been that development of double-stable, or triple-stable in the cases where the tTS was also used, cell lines expressing all the necessary Tet-On system components in satisfactory levels was particularly time-consuming, labour intensive and technically challenging. Transduction of the

cell lines with three separate plasmids expressing rtTA, tTS and TRE-GALV was inefficient, and screening of a large number of subclones was necessary to obtain a few that exhibited very stringent control of GALV expression. The limitations of DNA transduction procedures significantly restrict the use of the two-plasmid-based (or three-plasmid-based) Tet system. Sequential transduction of the cell lines means that the laborious screening process needs to be repeated two or three times, adding considerable length (in the range of months) to the time required to obtain the final cell lines. To overcome these problems several groups have incorporated the Tet system into viral vectors that can be produced at high titer and infect cells with very high efficiency, allowing for more efficient and rapid generation of populations of cells with regulatable gene expression (Bohl, Naffakh et al. 1997; Hu, Ji et al. 1997; Bohl, Salvetti et al. 1998; Corti, Sabate et al. 1999; Corti, Sanchez-Capelo et al. 1999; Pitzer, Schindowski et al. 1999; Kafri, van Praag et al. 2000). Generation of stable cells lines such as the ones described in this thesis requires stable, sustained gene expression. Retroviral or lentiviral vectors expressing the different components of the Tet system would therefore be best suited for this application. Several groups have developed all-in-one retroviral vectors containing the two key component of the Tet system, the transactivator and the TRE-gene. Application of such vectors in the generation of stable cell lines expressing GALV would considerably reduce the length of time and labour required to obtain the final product and would allow the application of this system to a wider range of cells. Within the time course of this thesis it was not possible to develop suitable viral vector expressing GALV under the control of the Tet-On system for use in generating stable cell lines, although this represents a critical part of the future work arising from this thesis.

8.4 Conclusion

In conclusion, work presented in this thesis describes a way to generate effective allogeneic tumour cell-containing vaccines, capable of inducing potent protective as well as therapeutic antitumour immunity. Of the three different gene modifications (cytokine expression, hsp expression and FMG-mediated fusion) investigated for their potency to augment the therapeutic potential of an allogeneic-based tumour cell vaccine in a mouse model of melanoma, FMG-induced fusion is shown to be the most effective one. We have begun analysing the important features that make the

allogeneic-containing FMG-fused tumour cell vaccines potently immunogeneic and set the ground for further, more detailed, characterisation of the key molecular cellular responses supporting this effect. Finally, we have developed a strategy to generate relevant human tumour cell vaccines for clinical use using a regulated gene expression system.

8.5 Future Work Stemming From This Thesis.

1. Comparison of effectiveness of fusing tumour cell vaccines when different melanoma allogeneic cell lines (such as the S91-M3 melanoma) are used as the allogeneic partner in the allogeneic/syngeneic vaccines.
2. Comparison of the effectiveness of fusing tumour cell vaccines in different melanoma models, e.g. when K1735 are used as the autologous tumour in C3H mice or when S91-M3 cells are used as the autologous tumour in DBA mice.
3. Determine whether the allo-effect can be incorporated into syngeneic tumour vaccines to enhance immunogenicity, for example modify B16 tumour cells to co-express VSV-G and D^k and test as a vaccine against B16 challenge.
4. Investigation of the role of FMG-mediated fusion of tumour cells with DCs in the efficacy of such hybrid vaccines. Fusion hybrids between tumour cells and DCs have been successfully used in preclinical and clinical studies to induce antitumour immunity (Gong Jianlin 1997, May; Wang, Saffold et al. 1998; Gong, Nikrui et al. 2000; Kugler, Stuhler et al. 2000).
5. Extended characterisation of the A375 parental and Tet system modified cell lines and development of a protocol for the efficient fusion of these allogeneic melanoma cell lines with patient derived material.
6. Generation of viral vectors expressing rtTA, TRE-GALV and tTS for application in the development of stable cell lines expressing GALV.
7. Investigation of the effectiveness of FMG-fusing allogeneic/syngeneic human tumour cell vaccines in Phase I/II clinical trials.

The following publications were derived from this thesis or contain work to which I have contributed:

1 **Linardakis E**, Bateman A, Phan V, Ahmed A, Gough MJ, Olivier K, Kennedy R, Harrington K, Melcher AA, Vile RG (2002). Enhancing the Efficacy of a Weak Allogeneic Melanoma Vaccine by Viral Fusogenic Membrane Glycoprotein-mediated Tumor Cell – Tumor Cell Fusion. *Cancer Research* Oct 1; 62(19):5495-504.

2 Bateman A, Harrington K, Kottke T, Ahmed A, Melcher AA, Gough MJ, **Linardakis E**, Riddle DS, Dietz A, Lohse CM, Strome S, Peterson T, Simari R, Vile RG (2002). Viral Fusogenic Membrane Glycoproteins Kill Solid Tumor Cells by Nonapoptotic Mechanisms That Promote Cross Presentation of Tumor Antigens by Dendritic Cells. *Cancer Research* Nov 15; 62(22):6566-6578.

3 Gough MJ, Melcher AA, Ahmed A, Crittenden MR, Riddle DS, **Linardakis E**, Ruchatz AN, Emiliusen LM, Vile RG (2001). Macrophages Orchestrate the Immune Response to Tumor Cell Death. *Cancer Research* Oct. 1; 61: 7240-7247

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Anti-tumour activity against B16-F10 melanoma with a GM-CSF secreting allogeneic tumour cell vaccine

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